Addressing current health issues confronting warm water culture of yellowtail kingfish

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Staff at the University of Western Australia's Pharmacy Department, Professor Lee Yong Lim and Dr Edith Tang contributed greatly to the aspect of the project on fluke management through the formulation and preparation of beads containing praziquantel. A UWA Pathfinder Grant contributed to the PZQ efficacy trial.

Executive Summary

This project addressed a number of key issues associated with the culture of yellowtail kingfish (*Seriola lalandi*, YTK) in warm water, including optimising the use of hydrogen peroxide (which is more toxic in warm water), investigating alternatives to the management of monogenean flukes (*Neobenedenia girellae* and *Zeuxapta seriolae*) in warm water and several aspects relating to a better understanding of the bacteria which cause disease in YTK, including an assessment of an industry-funded autogenous vaccine against the pathogenic bacteria *Photobacterium damsellae* subspecies *damsellae*.

Our study investigating the effect of hydrogen peroxide dose and water temperature has been submitted to the journal Aquaculture. In summary, this study demonstrated that whilst 340 mg/L of hydrogen peroxide is close to the lethal limit for YTK in warm water, very few of the measured physiological responses in the fish were able to predict this. Lower doses of hydrogen peroxide remain effective and carry a wide safety margin. Acknowledging this information, industry have subsequently modified their commercial bathing protocols for YTK.

Whilst these modified bathing practices provide a greater safety margin for the fish, they remain labour intensive and costly to perform. Work conducted under this project to investigate alternatives to peroxide bathing included the use of herbal extracts, freshwater bathing and further work on palatable praziquantel. In collaboration with the Tokyo University of Marine Science, Mr Jack Ingelbrecht was awarded first class honours for his work on herbal extracts to manage monogenean flukes. He demonstrated that both garlic and rosemary extracts were effective at reducing recruitment of *Zeuxapta* oncomiracidia and treating existing infections of this parasite. This work has been accepted for publication in the journal Aquaculture. Ms Joan Gao demonstrated that garlic was not effective at reducing recruitment of *Neobenedenia* oncomiracidia. Based on this work, the use of herbal extracts will be investigated in further detail in collaboration with industry under FRDC2017-030.

Ms Joan Gao also demonstrated that whilst freshwater (0 ppt) resulted in measureable, negative physiological impacts on YTK after 30 and 60 minutes of bathing, salinities of 2 and 5 ppt were very well tolerated for these bathe periods. Mr Ingelbrecht subsequently demonstrated that salinities of 2 and 5 ppt are effective at killing both *Zeuxapta* and *Neobenedenia in vitro*. To date, this work suggests that low salinity bathing could be a viable option for treating monogeneans in YTK once the industry grows to a size where well-boats are being used. To conclude this work a final trial is required in which low salinities are tested *in vivo* with fluke infected fish. Once this trial has been conducted this work will be prepared for publication.

In collaboration with the Pharmacy Department of the University of Western Australia we have formulated, manufactured and tested the palatability, digestibility and efficacy of beads containing the proven anthelminthic agent, praziquantel. Results of these trials are very encouraging and these formulations appear to be the most palatable, digestible and efficacious forms of praziquantel we have tested since our work in this field began 10 years ago. Work will continue on this encouraging formulation, including investigating options for IP protection.

A preliminary assessment of the effectiveness of the current industry-funded autogenous vaccine against *Photobacterium damsellae* subspecies *damsellae* was completed under this project. We demonstrated that YTK receiving the vaccine responded in terms of increased antibody production against the strains of this bacteria within the vaccine, demonstrating that it is effective against those strains.

Under this project Ms Nipa Gupta characterised 16 strains of *Photobacterium damsellae* subspecies *damsellae.* This work has demonstrated significant differences between Photobacterium strains based on the presence of virulence plasmids and haemolysis on both horse and YTK blood agar plates. These genetic and phenotypic differences result in significant differences in the virulence of these strains to YTK based on challenge trials we have conducted under this project. This work was completed after development of the aforementioned autogenous vaccine currently being used by industry and importantly this vaccine does not contain these more virulent strains. This work is now continuing in collaboration with Dr Andrew Barnes (University of Queensland) under the FRDC vaccine project “2018-101 A trivalent vaccine for sustainable Yellowtail Kingfish growout” and this work will be very useful in assisting to identity appropriate antigens for the new vaccines to be developed under that project.

The findings of this project have been conveyed to industry and those aspects of the project that have commercial relevance are being adopted or investigated further by both industry and our research partners.

Keywords

Yellowtail kingfish, *Seriola lalandi*, parasitism, monogeneans, flukes, *Zeuxapta seriolae*, *Neobenedenia girellae,* hydrogen peroxide, praziquantel, palatability, herbs, garlic, rosemary, disease, bacteria, *Photobacterium damsellae, Vibrio harveyi,* autogenous vaccine, vaccine efficacy.

Introduction

Yellowtail kingfish (*Seriola lalandi*, YTK) farming is identified nationally as the greatest opportunity for new aquaculture development in the next few decades through substantial increases in farmed area. This will deliver substantial increase in product to market, and increased use of locally produced aqua feeds, resulting in growth in regional economies and employment. Within 10 years, YTK production is expected to increase by 34,000 tonnes, worth $440 million, and using 68,000 tonnes of aqua feed worth $136 million (Stone et al. 2018).

The culture of this species in warm water has many advantages, yet creates some unique challenges. Managing the monogenean skin fluke *Neobenedenia girellae* and monogenean gill fluke *Zeuxapta seriolae* in the warm water environment, for example, is currently a major cost burden on the YTK industry due to loss of growth and mortality; and because the rapid life cycle duration of these monogenean parasites in warm water requires more frequent bathing. Moreover, the standard management practice of bathing in hydrogen peroxide carries a greater fish mortality risk in warm water and must therefore be optimised and more carefully managed. Whilst some research has been conducted previously on peroxide optimisation for YTK, this information has not been made available to YTK farmers in Western Australia (WA) and has had a significant financial impact on the fledgling WA industry. Bacterial diseases are also problematic in warm water YTK farming. Photobacteriosis caused by *Photobacterium damselae* ssp *damselae* is a recurring problem in warm water culture and outbreaks of this disease are often associated with *Vibrio harveyi*. An autogenous vaccine has been developed for Photobacterium, but its efficacy had not been tested experimentally.

This project was therefore developed to assist industry in overcoming some key short-term health management constraints that could potentially limit industry expansion in the warm water environments of WA. These include the use of hydrogen peroxide in warm water, investigating alternative options to hydrogen peroxide for managing flukes and developing a greater understanding of the bacteria *Photobacterium damselae* ssp *damselae* that has been implicated in mortality events in WA.

Objectives

The following objectives were agreed in the contract, with the exception of Objective 4 which was added later to assist with meeting Objective 5.

1. Optimise the use of hydrogen peroxide to treat flukes in warm water.
2. Investigate alternative fluke management methods to hydrogen peroxide in warm water.
3. Quantify the benefits of an autogenous vaccine against *Photobacterium damselae* subspecies *damselae*.
4. Characterise Australian strains of *Photobacterium damselae* subspecies *damselae*.
5. Determine whether interactions exist between *Photobacterium damselae* subspecies *damselae* and *Vibrio harveyi* that influence virulence.

Methods

This methods section is arranged according to the aforementioned objectives.

1. *Optimise the use of hydrogen peroxide to treat flukes in warm water*

The methodology used in the study is detailed in the appended manuscript (Appendix 4).

In brief, this objective was addressed through a repetitive bathing study in which healthy, parasite-free YTK (average 116 grams) were treated with four hydrogen peroxide doses (0, 85, 170 and 340 mg/L) at two temperatures (19°C and 27°C) on two occasions, 14 days apart. The effect of these treatments on the fish was assessed using a range of parameters including survival, histology (both standard histology and mucous mapping (Quantidoc™)), mucus lysozyme and several blood parameters including haematology, blood biochemistry, osmolality, albumin oxidation (Inflamark™) and serum lysozyme.

2. *Investigate alternative fluke management methods to hydrogen peroxide in warm water*

This objective was addressed through three sub-components; i) dietary herbs and herbal extracts as prophylactics and therapeutics against monogenean flukes ii) freshwater bathing and iii) further investigations into palatable praziquantel.

2.i. Dietary herbs and herbal extracts as prophylactics and therapeutics against monogenean flukes

This aspect of the study was conducted through two student projects. Curtin University Master's student Joan Gao completed a thesis titled "The effects of garlic supplemented diets on *Neobenedenia girellae* attachment success on *Seriola lalandi*" and Murdoch University student Jack Ingelbrecht completed a First Class Honours thesis titled "Anthelmintic herbal extracts as potential prophylactics or treatments for monogenean infections in cultured yellowtail kingfish (*Seriola lalandi*)"

Both theses are appended to this report (Appendix 5 and 6) and contain the detailed methodology used in each study. Mr Jack Ingelbrecht's study has also been accepted to the journal Aquaculture (Appendix 7).

In brief, Ms Gao investigated the preventative effects of dietary supplementation of commercially available garlic powder (Spencers, Anchor Foods) at 10 and 20 g/kg and two commercial garlic derivatives Aquagarlic-P and Aquagarlic- A, both at 2 g/kg (manufacturers recommendation) as prophylactics against *Nenobenedenia girellae* infection *in vivo*. The supplemented diets were fed to juvenile YTK for 8 weeks before challenging them with 30 *Neobenedenia* oncomiracidia per fish. Flukes were allowed to grow on the fish for 6 days before being removed via freshwater bathing and counted to quantify attachment success.

Mr Ingelbrecht's study comprised two *in vivo* studies following a preliminary *in vitro* Petri dish study. The first *in vivo* study investigated the prophylactic effects of the same aforeomentioned natural garlic powder (10 g/kg) and Aquagarlic-P (2 g/kg) in addition to an ethanolic rosemary leaf extract (168 mL/kg). This treatment arose following a meeting and subsequent collaboration agreement with Professor Masashi Maita

during a state government funded knowledge quest to Japan awarded to IOFA Technical Manager Justine Arnold. Diets containing these products were fed to YTK (average 148 grams) for 30 days before challenging with *Zeuxapta seriolae* oncomiracidia (50 oncomiracidia per fish). Following challenge, fish were fed for a further 14 days on unmedicated diets to allow time for the parasites to grow before they were removed via praziquantel bathing and counted.

Mr Ingelbrecht's second *in vivo* study looked at the therapeutic effects of feeding diets containing herbs to YTK already infected with *Zeuxapta seriolae*. Three treatment diets and one unmedicated control diet were included. Based on the results of the first trial and in order to investigate a more commercially relevant form of rosemary, Aquagarlic-P was replaced with a commercial rosemary oil (essential rosemary oil, Range Products, Welshpool, Australia). The same ground garlic and ethanolic rosemary extract treatments and inclusion levels tested in Trial 1 were included. Treatment diets were prepared by mixing commercial YTK diets (9 mm Pelagica, Ridley Agriproducts, Narangba, Australia) with 15 mL/kg of fish oil for 10 minutes with the relevant quantity of the herbal treatment. Control diets were also mixed with 15 mL/kg of fish oil. These diets were fed to YTK (average 700 grams) for 20 days in triplicate tanks. After 10 days half of the fish in each tank were sampled by bathing them in praziquantel and counting collected flukes. The remaining fish were sampled using the same procedure at 20 days.

2.ii. Freshwater bathing

Freshwater bathing is an effective treatment against many ectoparasites and may be effective for removing monogenean flukes from YTK once the industry develops to the stage of using well boats. The first component of this study was conducted by Ms Joan Gao during her Master's year to determine the effect of salinity, fish size and bathe duration on the physiology of healthy, uninfected YTK to determine how well they tolerate salinities of 0, 2 and 5 ppt. The detailed methods are given in the appended student report (Appendix 8). The second aspect was an *in vitro* study conducted by Mr Ingelbrecht to determine the time required to kill flukes at these salinities and whether an overlap occurs between the salinity in which fluke are effectively killed and which YTK tolerate well.

Under Part 1, Ms Gao bathed fish (large = average 2.9 kg or small = average 362 g) at four salinities (0, 2, 5 and 35 ppt) for three durations (10, 30 and 60 mins) in triplicate. At the end of each bathe period, fish were anaesthetised and blood sampled for measurement of plasma osmolality, individual blood electrolytes and plasma protein. Plasma cortisol was also measured in the most extreme treatments (0 and 35 ppt after 60 minutes).

Under Part 2 of this project, Mr Ingelbrecht exposed 30 newly hatched *Neobenedenia* oncomiracidia, and 30 mature *Neobenedenia* and *Zeuxapta* to the same salinities used in Part 1 in triplicate 6-well culture plates (due to insufficient numbers, mature *Neobenedenia* flukes were not subjected to 0 ppt). Parasites were monitored constantly using a light microscope and time to death was recorded when 100% of parasites in each replicate stop moving. The time to the first parasitic mortality was also recorded to determine the range in time of mortalities.

2.iii. *Palatable praziquantel*

Staff at the Pharmacy Department at the University of Western Australia (UWA) have developed and patented a preparation for taste-masking active pharmaceutical ingredients for oral delivery. The method was developed for masking the bitter taste of medications such as midazolam for children. Under this project we assessed the ability of this formulation (background IP to this project) and new formulations (new IP) to mask the bitter taste of the proven anthelminthic agent, praziquantel (PZQ) in YTK.

UWA initially provided ACAAR with small quantities of 23 different types of beads containing PZQ. The beads were assessed by ACAAR staff purely based on their smell. Based on this assessment, UWA then formulated and prepared a number of types of beads which were assessed *in vivo*. Four palatability and digestibility trials were conducted with YTK in which the formulation of the beads was adjusted in each successive trial based on the outcome of the previous trial. The treatments tested in each trial are shown in Table 1. Following these palatability and digestibility assessments, an efficacy trial was conducted at DPIRD's Geraldton Annex in which the two most promising formulations (UWA C and UWA B) were fed to fluke infected fish (Trial 5, Table 1). This efficacy trial was co-funded by a UWA Pathfinder grant.

In all trials, PZQ beads were incorporated into fish food by mixing the beads with ground, commercial YTK feed and reconstituting into pellets using a Dolly pasta maker and air dried (Figure 1). Control diets containing either no PZQ or pure PZQ powder were tested in most trials depending on tank availability (Table 1). The efficacy trial included both a pure PZQ treatment and non-medicated control. All PZQ diets contained the equivalent of 10 grams/kg of pure PZQ.

Figure 1: Pellets made with Dolly pasta maker for palatability and efficacy trials. Note white PZQ beads in the right hand photo.

Table 1: Treatments tested in each palatability trial.

Trial	Fish size	Fish per tank	Treatment	Loading rate (w/w)
1	172	10	UWA1	68%
			UWA2	62%
			UWA3	74%
			UWA4	74%
			Pure PZQ control	100%
			no PZQ control	-
2	720	8	UWA5	86%
			UWA6	86%
			UWA7	86%
			UWA8	86%
			UWA9	86%
			no PZQ control	-
3	175 vs 2000	8 small	UWA A	82%
		2 large	UWA E	81%
			no PZQ control	-
4	263	10	UWA B	75%
			UWA C	78%
			UWA D	84%
			no PZQ control	-
5	1600	8	UWA B	78%
			UWA C	80%
			Pure PZQ control	-
			no PZQ control	-

In Trials 1 to 4, palatability was tested over 5 days in unreplicated 1.5 m^3 tanks each containing YTK of the sizes shown in Table 1. In Trial 5, fluke-infected fish were brought from an IOFA seacage and were randomly distributed into 12 x 4.5 m^3 tanks (3 replicate tanks per treatment) and acclimated to the experimental conditions for two days, during which time they were fed the non-medicated control diet before switching to the treatment diets which were fed for 6 days.

In each trial, fish were offered a fixed ration based on their weight and water temperature in a single morning feed over a maximum duration of three minutes. When the entire ration was consumed within this time, the time taken to consume it was recorded. The data from Trials 1 to 4 were analysed with days as replicates. A simple assessment of the digestibility of the beads was made at the end of Trials 1 to 4 by dissecting the fish and inspecting their stomach and digestive tract for whole beads. At the conclusion of Trial 5, fish in each tank were bathed in PZQ and the total number of flukes from the pooled batch of fish was counted to assess efficacy. Efficacy of the medicated diets was measured as the percentage reduction in fluke numbers relative to the unmedicated control diet.

3. *Quantify the benefits of an autogenous vaccine against* Photobacterium damselae *subspecies* damselae.

This aspect of the study was conducted in collaboration with Associate Professor Andrew Barnes and Honours student Mr Jay Anderson from the University of Queensland. Two trials were conducted to quantify the benefits of vaccinating fish with an industry-funded autogenous vaccine against *Photobacterium damselae* ssp *damselae* (Pdd). This vaccine contained two strains of Pdd (QMA0509 (=AS-15-3942#7) and QMA0510 (=AS-15-3942#8)) isolated from diagnostic cases in WA and characterised via NextGen sequencing by Andrew Barnes. QMA0509 and QMA0510 were designated previously as high and low virulence by Dr Nicky Buller's lab based on the degree of haemolysis shown on horse blood agar.

The first preliminary trial compared the efficacy of the vaccine in a small group of fish held at DPIRD's Geraldton Annex where they were likely to be exposed to these bacteria. Fish (average 67 grams) were vaccinated at ACAAR in Fremantle on January 30th 2017 and immediately transferred to the Geraldton Annex. Fish were bled on 26th April (i.e. after 84 days) for determination of antibody response. The water temperature at the Annex during this time ranged from 23.0 to 29.1°C with an average of 25.7°C. Based on this average temperature, the number of degree-days (dd) between vaccination and sampling was therefore 2160 dd.

In the second trial, we measured the antibody response to the vaccine by comparing non-vaccinated, single vaccinated and booster vaccinated fish. Fish allocated to the boost treatment received the second vaccination 94 days after the primary vaccination. Fish were sampled on days 3, 8, 11, 15, 63, 94 and 107 days post primary vaccination. Multiple sampling points were taken in control and single vaccinated fish prior to the boost in order to track the maturation of the primary vaccination and compare it with the control fish who were likely to have been naturally exposed to Pdd and therefore acquiring antibodies against it.

The methods used for quantifying vaccine efficacy were the same in both trials. Whole cell ELISA was conducted. Firstly, ELISA plates were coated with whole cell antigen as follows. Pdd strains QMA0509 and QMA0510 were recovered from frozen stocks on tryptone soy agar + 2% NaCl (TSA2) and grown overnight at 22°C. A single colony was picked and used to inoculate 10 mL TSB2 and grown with aeration overnight. One millitre of this culture was then used to inoculate 100 mL fresh TSB2 (flask:medium ratio =5:1) at 22°C and incubated overnight with shaking. The overnight culture was chilled rapidly on ice to < 4°C then inactivated for 48 h at 4°C by addition of 0.5 mL formalin solution (40%). After inactivation, cells were harvested by centrifugation, the supernatant discarded and the pellet resuspended to OD600 = 1.5 in carbonate-bicarbonate buffer, pH 9. The suspension was distributed 100 µL per well into 96 well high-binding Microlon ELISA plates (Greiner BioOne, Germany). Half of each plate (all wells in columns 1-6) was coated with QMA0509 inactivated cells and the second half (wells in columns 7-12) coated with QMA0510. Plates were briefly centrifuged at 500 x g with no brake and then incubated overnight at 4°C. The liquid was then removed and the plates tapped dry on absorbent paper. Plates were wrapped and frozen at -20°C until needed.

Antibody response to Pdd was determined as follows. Plates (defrosted at room temperature) were blocked with 1% bovine serum albumin dissolved in tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4°C. The blocking reagent was removed and plates dried by tapping on absorbent paper. Wells were washed three times with 100 µL each TBST. YTK antiserum was diluted 1:32 in cold TBST and 100µL/well incubated at room temperature (RT) for 3 h. Plates were thrice washed in TBST and specific antibody was detected with sheep vs barramundi IgM secondary antibody that cross-reacts with kingfish IgM (Fielder, Landos, Barnes, unpublished), diluted 1:6000 in TBST and incubated for 1h at RT. After washing a further 3 times in TBST, binding secondary antibody was detected with donkey Vs sheep IgG alkaline phosphatase conjugate (1:15,000 in TBST). After 1 h at RT, unbound conjugated antibody was removed via three washes with TBST followed by a single wash in TBS and colour was developed by addition of 50 µL per well of p-nitrophenylphosphate liquid substrate (Sigma) for 1 h at room temperature in the dark. Absorbance was read at 405 nm with a BMG Fluostar Optima plate reader.

Technical replicate means were imported from Excel into GraphPad Prism™ in which ELISA OD for individual fish receiving control, primary or primary then booster vaccination was plotted against sampling time (Satpathy et al., 2001). Means and standard deviations were calculated per treatment per time and plotted as bars while retaining the individual values in the plots to permit identification of high and low responding fish. For comparison of control vs primary and control or primary vs boost, data were analysed for normality using Levene's test, then ANOVA was used to detect significant differences between treatment groups within each time point.

4. *Characterisation of Australian strains of Photobacterium damselae subspecies damselae.*

To date, the bacteria *Photobacterium damselae* subspecies *damselae* has generally been considered a secondary, opportunistic pathogen in Australia, however evidence exists overseas of strains which harbour virulence genes associated with primary pathogenicity (Labella et al., 2010; Rivas et al, 2013). This aspect of the project forms part of a PhD project being undertaken by Ms Nipa Gupta at Murdoch University. Ms Gupta has compared 16 isolates of *Photobacterium damselae* subspecies *damselae* from different parts of Australia. The majority of isolates have been from YTK, however for comparative purposes two Pdd isolates tested were from pink snapper (*Pagrus auratus*). The strains characterised included those two included in the aforementioned vaccine and several which have been sequenced by Professor Barnes. A summary of the strains investigated and their origins are shown in Table 2. These strains were phenotypically characterised using a wide range of factors detailed in Table 3 and subsequently categorised into biotypes based on these results. Haemolytic activity of each strain was measured on both horse blood and YTK blood as the diameter of the haemolytic zone. The presence of virulence genes pPHDD1, dly, hlyApl, hlyAch, and PlpV were determined by PCR with specific primers and correlated with biotype and haemolytic activity.

Table 2: DPRID- isolate number, host species and origin of the *Photobacterium damselae* subspecies *damselae* strains characterised.

Pdd isolates	Host species	Origin	Year of isolation
AS-15-3942#8	YTK	WA	2015
AS-15-3942#9	YTK	WA	2015
AS-15-3942#7	YTK	WA	2015
AS-16-0963#1	YTK	WA	2016
AS-16-0963#3	YTK	WA	2016
AS-17-6307#26	YTK	WA	2017
AS-17-6307#61	YTK	WA	2017
AS-17-6307#69	YTK	WA	2017
AS-17-7468#8	YTK	SA	2017
AS-17-7468#12	YTK	SA	2017
AS-18-0902#12	YTK	SA	2018
AS-18-0902#17	YTK	SA	2018
AS-14-1386#1	Pink snapper	WA	2014
AS-16-2454#19	Pink snapper	WA	2016
AS-18-2495#3	YTK	WA	2018
AS-18-2495#5	YTK	WA	2018

YTK= Yellow tail kingfish, WA= Western Australia, SA= South Australia

Table 3: Phenotypic characteristics by which *Photobacterium damselae* subspecies *damselae* isolates were categorised into biotypes.

Phenotypic Characteristics
Colony colour
Swarming activity
Growth at temperatures of 4, 24 and 37°C
Growth at salinities of 0 and 30 ppt
Bioluminescence
Urease
Sucrose
Trehalose
Methyl Red
Voges-Proskauer
Ornithine Decarboxylase
Lysine Decarboxylase
Arginine Decarboxylase
Sensitivity to Vibriostatic 0/129 compound
Dnase
Chitinase

To confirm that the presence of the haemolysin gene within the pPHDD1 plasmid is a major factor determining virulence, a challenge trial was conducted in which we compared the pathogenicity of a Pdd strain which contained this plasmid (AS-16-0963#3) (presence of pPHDD1, *dly* and *hlyApl* gene) and two plasmidless isolates, AS-16-0963#1 and AS-15-3942#7 (which both possess the chromosomal haemolysin gene $hlyA_{ch}$ but neither *dly* or *hlyApl* gene). The latter isolate was the same one used in challenge trials under FRDC project 2016-200.40 and which did not result in mortality. Bacterial isolates were cultured in tryptone

soya broth with 2% sterile NaCl (TSB-2) at 24°C for 6-7 h for log phase of growth. The cultures were dispensed into 1 mL amounts and stored in 20% sterile glycerol at -80°C until the infection trial. The viable bacteria (10^8 and 10^5 CFU/mL) in the suspension were confirmed through gradient dilution plate counting. Healthy, unvaccinated YTK (152 ±18 g) were transferred to the QAP facility at the Indian Ocean Marine Research Centre (IOMRC), at Waterman's Bay where they were stocked into flow through 300 L tanks at 21°C. Each of the three bacterial isolates were tested at two doses (10^7 and 10^4 CFU/fish) in triplicate via an IP injection against a control treatment (PBS injection). After injection, all fish were returned to their respective tanks and fed twice daily for a further 10 days with commercial feed. Fish health and condition were monitored twice daily and were euthanised as soon as clinical signs, including lethargic swimming, flared opercula, skin lesions and loss of equilibrium were observed.

5. *Determine whether interactions exist between* Photobacterium damselae *subspecies* damselae *(Pdd) and* Vibrio harveyi *(Vh) that influence virulence*

This objective was addressed through a number of avenues.

5.i. In-vivo *challenge trials*.

The first *in vivo* challenge trial was conducted prior to the strain characterisation described above. In this trial, juvenile YTK (23 grams) were challenged at the QAP facility at Waterman's Bay with either Pdd, Vh or an equal quantity of both bacteria. The Pdd strains used were equal quantities of the two Pdd strains used in the aforementioned vaccine (AS-15-3942 #9 (which is QMA511 (=509) and AS-15-3942 #8 (QMA510). The Vh strains were equal quantities of two diagnostic case isolates from YTK. The first was a non-swarming colony (AS-16-0547 #5; not a common finding for Vh) and the second a swarming colony (AS-16-4149 #1; common finding for Vh).

Six treatment concentrations of bacteria ranging from 10^3 to 10^8 CFU/mL in 10^1 increments were tested in triplicate by injecting 0.1 mL of the bacterial suspension into the body cavity of each fish (giving an administered dose of 10^2 to 10^7 CFU/fish). Each tank was stocked with 30 microchipped fish; 10 were injected with Pdd alone, 10 with Vh alone and 10 with an equal quantity of Pdd and Vh. Two control tanks each contained 30 fish individually injected with 0.1 mL of saline.

Fish were monitored daily for signs of infection as previously described and once any evidence of infection was noted the fish were removed and immediately culled. Water temperature in the QAP tanks during the trial ranged from 15.5 to 18.0°C and averaged 17.0°C.

After 10 days, a low oxygen stress was applied to fish in all remaining tanks. The 300 L tanks were drained to 50 litres. Dissolved oxygen in this volume subsequently dropped from 100% saturation to 30-40% saturation where it was maintained for 30 minutes before refilling the tanks with saturated water. Following this stress, fish were monitored for a further 5 days.

Following the aforementioned strain characterisation another trial was conducted which compared mortality in fish challenged with either Pdd (plasmid bearing AS-16-0963#3) alone or in combination with *Vibrio harveyi* (AS-17-6320#3). The methods used in this trial followed that described above. Fish used in this trial were 218 ± 3.9 g and were challenged with 0.5 x 10^4 CFU/fish of each bacterium.

5.ii. Gene expression

An *in vitro* trial was conducted in which the expression of the *Vibrio harveyi* haemolysin gene (Vhh) was measured either in a monoculture culture of *Vibrio harveyi* (AS-18-2495#1) or in mixed cultures of this *Vibrio harveyi* strain with one of the five plasmid bearing Pdd strains.

For the quantification of mRNA, the RNA from bacterial cells grown in TSB was extracted with TRizol (Invitrogen™) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA with Quantitect Reverse Transcription kit (QIAGEN™), including a gDNA wipeout step. The cDNA was stored at -20ºC for further work. Primers from *Vhh* gene (target) and reference gene (rpoA) were taken from the literature (Defoirdt et al., 2007; Ruwandeepika et al., 2011; Ruwandeepika et al., 2011). RR-qPCR reactions were performed in a Rotor gene Q Real-Time PCR Detection System with an initial denaturation cycle of 95ºC for 5 min followed by 40 cycles of 95ºC for 5 s, 60ºC for 10 s. Amplification was followed by a standard melting curve from 65ºC to 95ºC to confirm that only one product was amplified and detected. The change in gene expression was normalized to the reference gene by calculating Ct value ($2^{-\Delta\Delta CT}$) according to (Livak & Schmittgen, 2001).

Results & Discussion

This section is arranged according to the aforementioned objectives.

1. *Optimise the use of hydrogen peroxide to treat flukes in warm water*

The results of this trial are detailed in the appended manuscript (Appendix 4). The most notable results are summarised here.

Survival was 100% in most treatments, with the exception of three fish which died in one warm-water, 340 mg L^{-1} replicate tank following the second bathe. Whilst there was no significant effect of peroxide dose or temperature on survival following repeated bathing, this mortality at the higher treatment dose in warm-water is still of commercial relevance as it suggests that this is very close to the lethal limit and that fish were more susceptible to this high dose in warm water and following repeat bathing.

Haematological analysis revealed no effect of temperature or hydrogen peroxide dose on the majority of haematology parameters. Whilst some blood biochemistry parameters were influenced by temperature, none were affected by peroxide concentration. This suggests that peroxide was not impacting osmoregulation in healthy, surviving fish. There were, however significant increases in plasma sodium ($P < 0.01$) and magnesium ($P = 0.02$) and considerable concomitant (but non-significant) increases in osmolality, chloride and potassium in the moribund fish treated at 340 mg L^{-1} following the second bathe compared to seemingly healthy fish in the same treatment (Table 4). The elevation in these parameters in moribund fish suggests disruption to the osmoregulation as a cause of morbidity in these fish.

Table 4: Electrolytes (mmol L^{-1}) and osmolality (mmol kg^{-1}) of plasma collected from healthy and moribund YTK following repeated hydrogen peroxide bathing at a peroxide dose of 340 mg L^{-1} held in warm water (26 °C)

Fish Status	Na	K	Cl	Ca	Mg	Osmolality
Moribund	210.3 ± 2.4	22.7 ± 8.1	193.0 ± 6.7	3.4 ± 0.1	4.7 ± 0.5	574 ± 40
Healthy	182.3 ± 8.2	15.2 ± 7.6	157.7 ± 6.1	3.4 ± 0.2	1.6 ± 0.7	502 ± 42
P	**< 0.01**	0.45	0.06	0.98	**0.02**	0.15

Serum and mucous lysozyme were unaffected by temperature, peroxide dose or the interaction of these terms. There was a significant effect of hydrogen peroxide concentration on the percentage of thiol oxidation in the plasma proteins ($P < 0.05$) (Figure 2). Fish bathed at 340 mg L^{-1} had significantly higher percentage of thiol oxidation (Inflamark®) than those bathed at 85 mg L^{-1} and in the control, demonstrating the sensitivity of this method for detecting oxidative stress in fish.

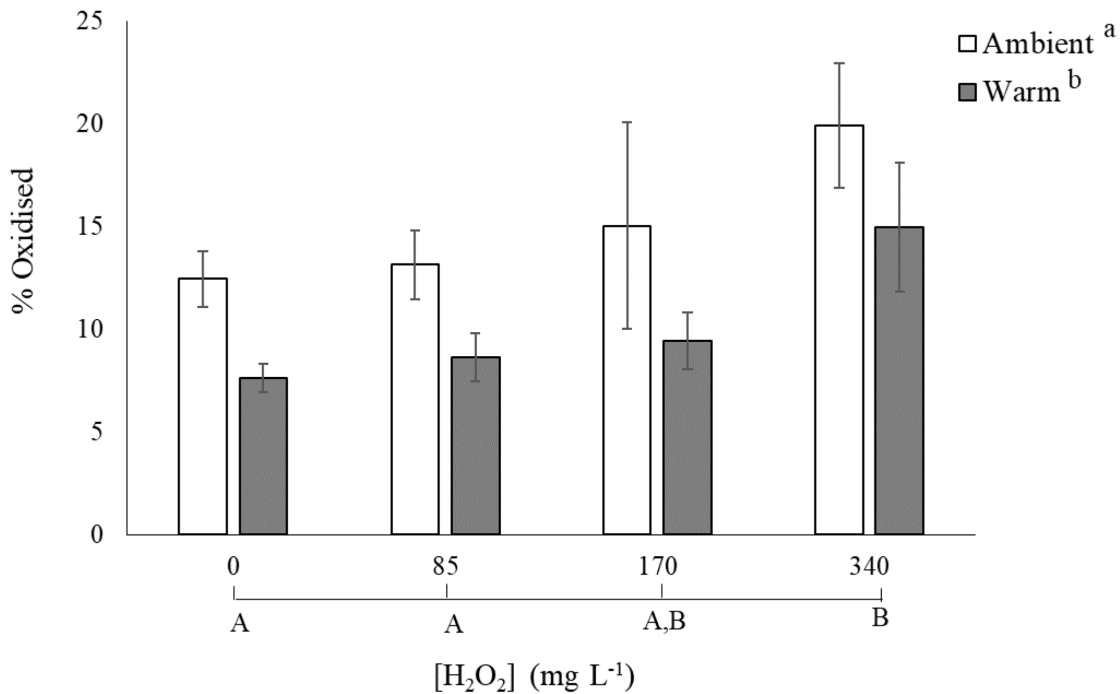


Figure 2: Oxidative stress measured as thiol oxidation of plasma protein (% oxidised) 48 hours after the second hydrogen peroxide (H_2O_2) bathe (14 days following the first bathe) conducted at ambient (19°C) and warm (27°C) water temperatures. Different uppercase letters denote significant differences among the peroxide concentration treatments, and different lowercase letters denote significant differences among water temperatures. Values are mean ± S.E.

Skin mucous cell mapping found peroxide dose but not temperature significantly reduced the size of the common mucous cells after the second bathe in fish treated at 340 mg L^{-1} compared to those in the control ($P = 0.01$) (Figure 3). The density of the common mucous cells (% of epidermis filled with mucus cells) was also significantly affected by peroxide dose, with the control fish having a significantly higher density of cells compared to those treated at the highest peroxide concentration ($P = 0.02$) (Figure 3).

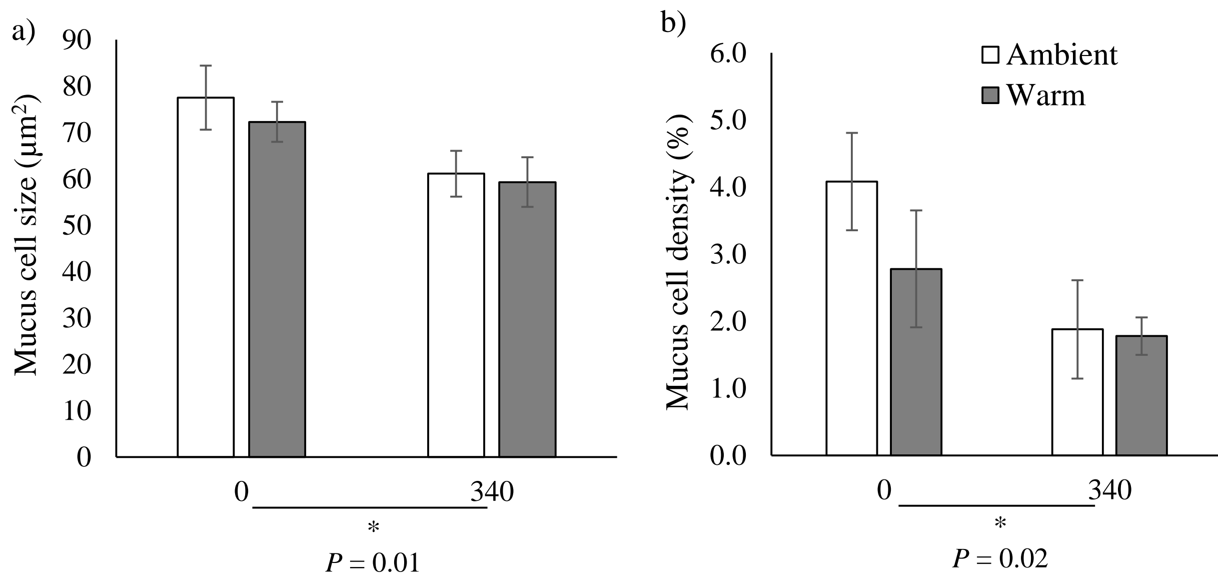


Figure 3: Effect of hydrogen peroxide dose (0 and 340 mg L^{-1}) and water temperature on a) the mucous cell size and b) density (as a % mucous to epithelium) within the skin epithelium of yellowtail kingfish subjected to repeated hydrogen peroxide bathing. Values are mean ± S.E.

Whilst hydrogen peroxide dose and water temperature did not impact skin thickness, the fish held in the peroxide treatment tanks had significantly thicker skin following the repeat bathing ($P < 0.001$). There was no significant effect of peroxide dose on the gill tissue in terms of the number of mucous cells per gill filament and no evidence of repeated bathing being detrimental to the number of mucous cells within gill tissue ($P = 0.123$). The number of mucus cells per gill filament were only significantly impacted by water temperature ($P= 0.002$), with those fish held in the ambient water temperature having significantly more mucous cells compared to those fish in the warm water.

Therefore, despite evidence that 340 mg L^{-1} of hydrogen peroxide for 30 minutes is very close to the lethal limit for YTK in warm water, this was not evident in many of the measured parameters, with the exception of thiol oxidation, mucus mapping data and some histological parameters. Whilst YTK appeared to be relatively tolerant of the high concentration of peroxide tested in the current study, these fish were not infected with flukes and any burden of fluke is likely to impact the overall health of the fish and make them more susceptible to the acute stresses of peroxide bathing.

The results also demonstrate that the lower concentrations of peroxide tested in the study had no measurable negative impact on the fish, regardless of temperature and repeat bathing. Industry have taken these findings on board and subsequently modified their bathing protocols to provide a wider safety margin against overdosing with hydrogen peroxide.

2. *Investigate alternative fluke management methods to hydrogen peroxide in warm water*

2.i. *Dietary herbs and herbal extracts as prophylactics and therapeutics against monogenean flukes*

The results of these trials are detailed in the appended theses of Ms Joan Gao (Appendix 5) and Mr Jack Ingelbrecht (Appendix 6) and in the appended manuscript that has been accepted for publication in Aquaculture (Appendix 7).

The most notable results from these studies are that garlic and rosemary were demonstrated to be effective as both prophylactics and treatments against *Zeuxapta* infections, but that garlic was not an effective prophylactic against *Neobenedia* infections (rosemary was not tested against *Neobenedenia* infections *in vitro*). As a prophylactic, recruitment was reduced by up to 80% and as a treatment parasite was reduced by up to 25%. The studies demonstrated that whilst these herbs were effective against *Zeuxapta,* they did not result in 100% protection or eradication and they will not therefore be a ‘cure all’ but worthy of inclusion within an integrated pest management scenario. There was also evidence that the ethanolic rosemary extract impacted negatively on growth. Based on these findings further research will be conducted into herbal extracts for fluke management under a revised FRDC2017-030.

2.ii. *Freshwater bathing*

The results of Part 1 of this aspect of the project conducted by Ms Joan Gao are detailed in the appended student report (Appendix 8). In summary, it was found that bathing YTK in freshwater (0 ppt) for 30 or 60 minutes resulted in significant, measureable physiological disturbances (but no mortality) in the fish. These disturbances were noticed as significant impacts on osmolality (Figure 4), individual electrolytes and plasma protein. Bathing at salinities of 2 or 5 ppt, however, resulted in no significant differences in any of these parameters compared with control fish bathed at 35 ppt. Cortisol levels measured after 60 minutes of bathing in freshwater or 35 ppt revealed considerable elevation at 0 ppt in both small and large fish, however these differences were not significant (Figure 5).

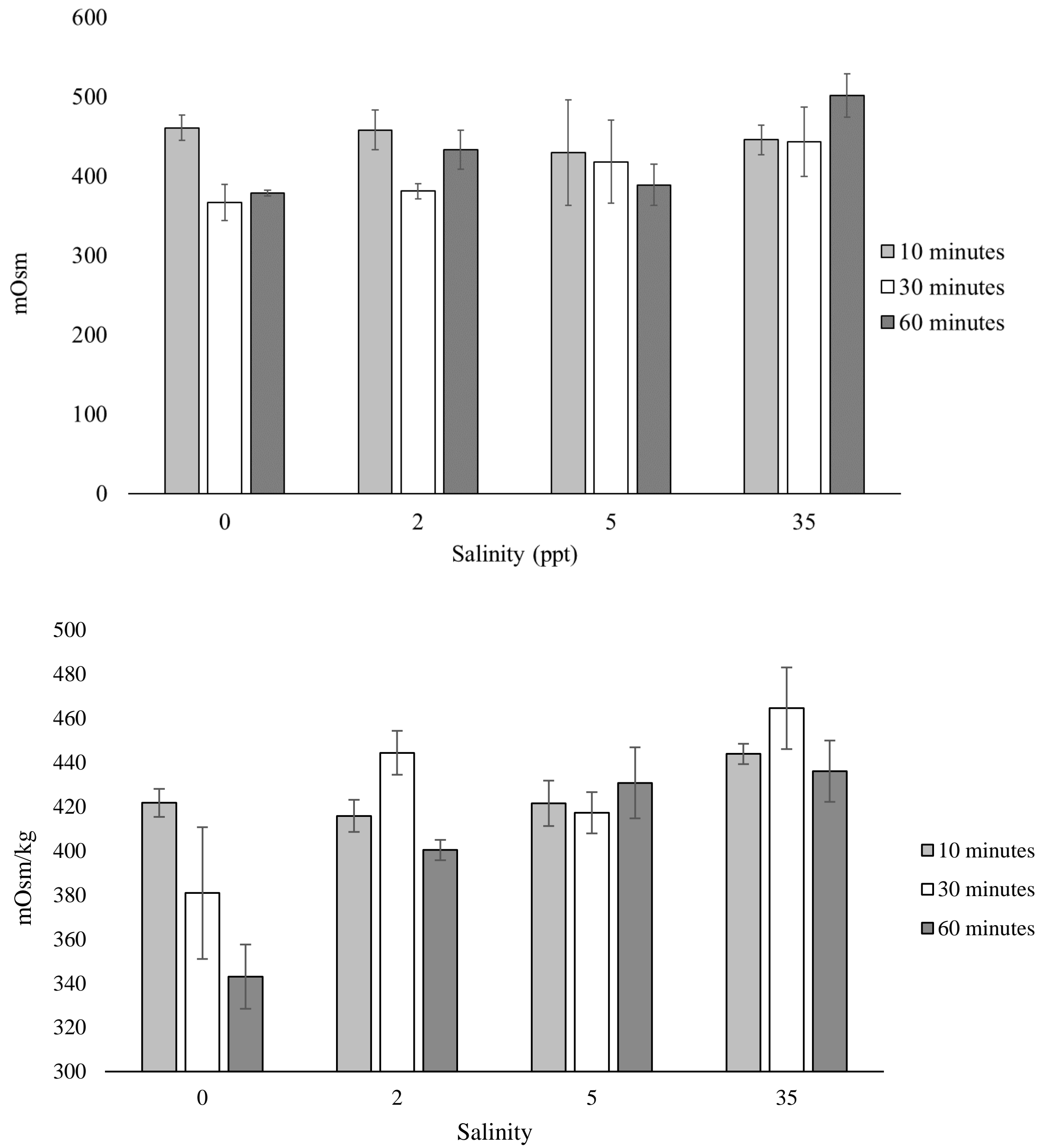
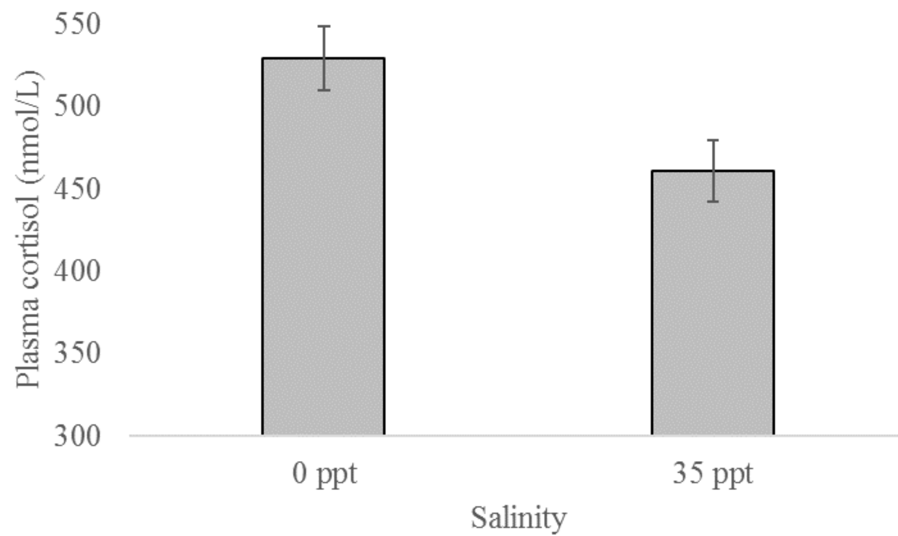


Figure 4: Plasma osmolality of small (average 362 g, top) and large (average 2.9 kg, bottom) YTK bathed at different salinities for different durations.



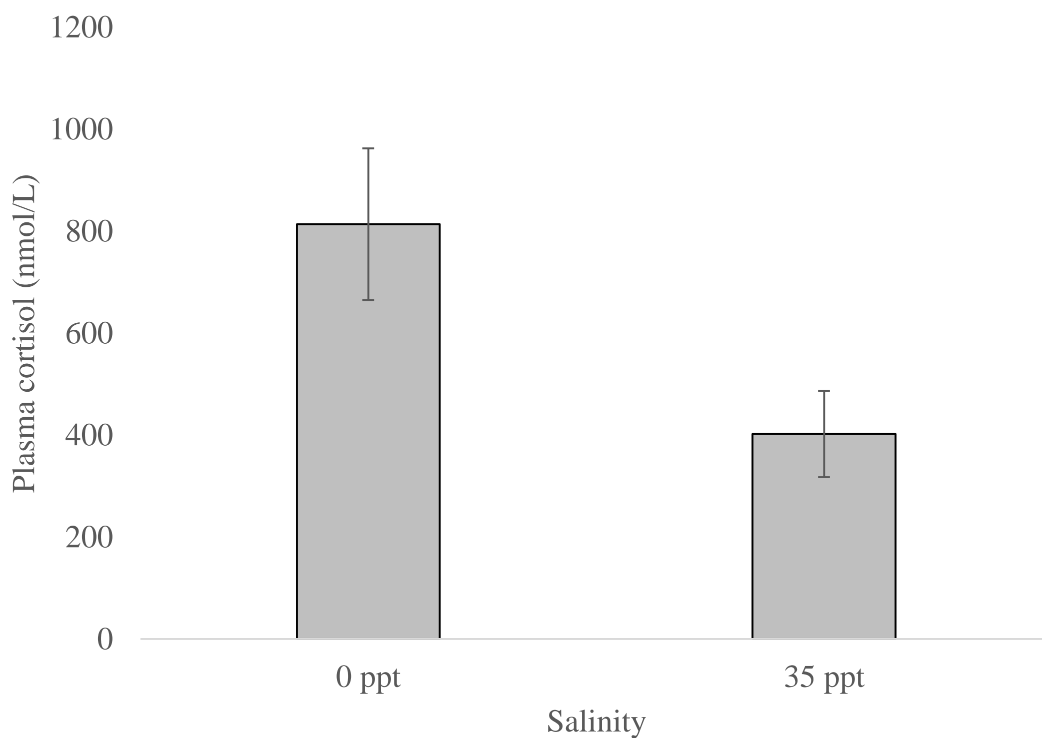


Figure 5: Plasma cortisol of small (average 362 g, top) and large (average 2.9 kg, bottom) YTK bathed for 60 minutes at 0 and 35 ppt.

In Part 2 of the project, Mr Ingelbrecht measured the time to 100% mortality of adult *Zeuxapta seriolae* and both adult and oncomiracidia of *Neobenedenia girellae in vitro* (Figure 6). Adult *N. girellae* were not exposed to freshwater due to a shortage of these flukes. Survival of flukes in control wells (35 ppt) was 100% after 36 hours and therefore these data are not shown. The results demonstrate that *Zeuxapta seriolae* adults and *Neobenedenia girellae* oncomiracidia are very susceptible to low salinity and 100% mortality of

flukes can be achieved in 10-15 minutes at 2 ppt. Time to 100% mortality was significantly longer at 5 ppt, but 100% mortality of these flukes could still be achieved within 30 minutes. Adults of *Neobenedenia girellae* were considerably more tolerant to low salinity and required 40 and 60 minutes of exposure for 100% mortality to occur at salinities of 2 ppt and 5 ppt, respectively.

These results suggest that bathing at 2 ppt should be well tolerated by YTK and that it should result in 100% removal of *Zeuxapta seriolae* adults and early stage *Neobenedenia girellae* within 20 minutes, but double this time to achieve 100% mortality of adult *Neobenedenia girellae*. Further research is required to determine if fluke tolerance to these low salinities is the same *in vivo* as it is *in vitro* (for example flukes may be more difficult to kill if they receive protection from the fish's mucous *in vivo*). Similarly, further work is required to determine if YTK are as tolerant to low salinity when they are already parasitised by monogenean flukes.

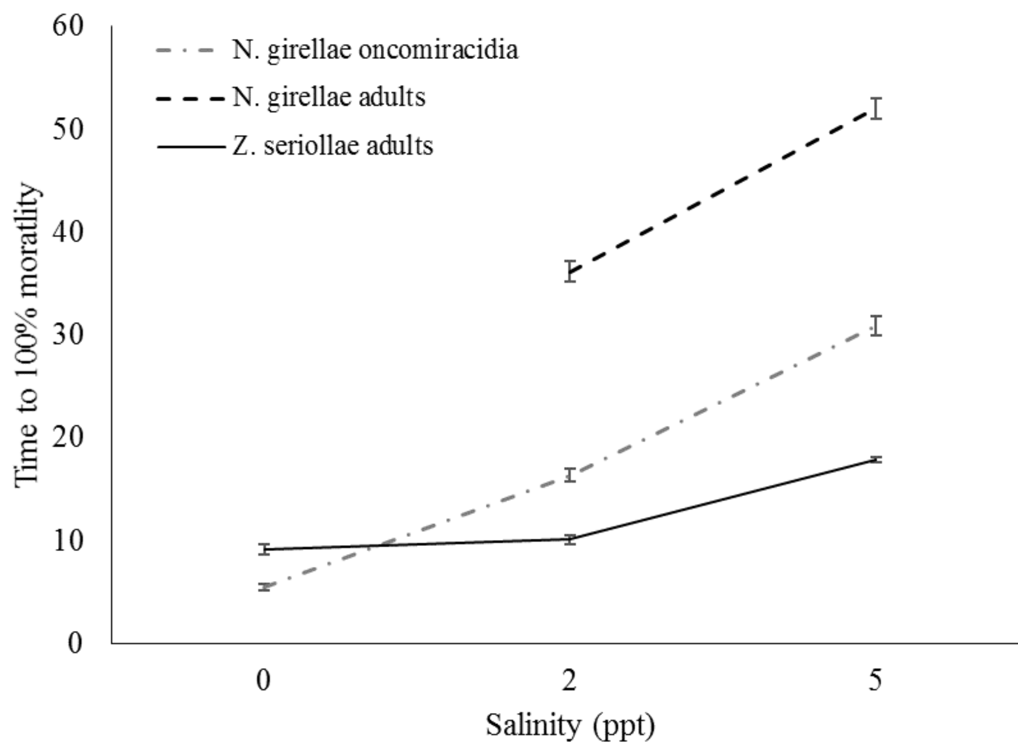


Figure 6: Time to 100% mortality of *Zeuxapta seriolae* (adults) and *Neobenedenia girellae* (oncomiracidium and adults) exposed to different salinities for different durations.

2.iii *Palatable praziquantel*

2.iv Palatable praziquantel Trial 1

There was a significant effect of diet on ingestion of the various feeds offered in Trial 1 (P<0.001) (Figure 7). Fish fed the diet containing pure PZQ consumed only 64% of the offered ration, significantly less than all other treatments. Fish in all other treatments ate ≥ 90% of their offered ration and there was no significant difference in ingestion between fish fed the unmedicated control and those fed the various UWA treatments. Fish in the unmedicated control treatment and the UWA3 treatment consumed 100% of the ration.

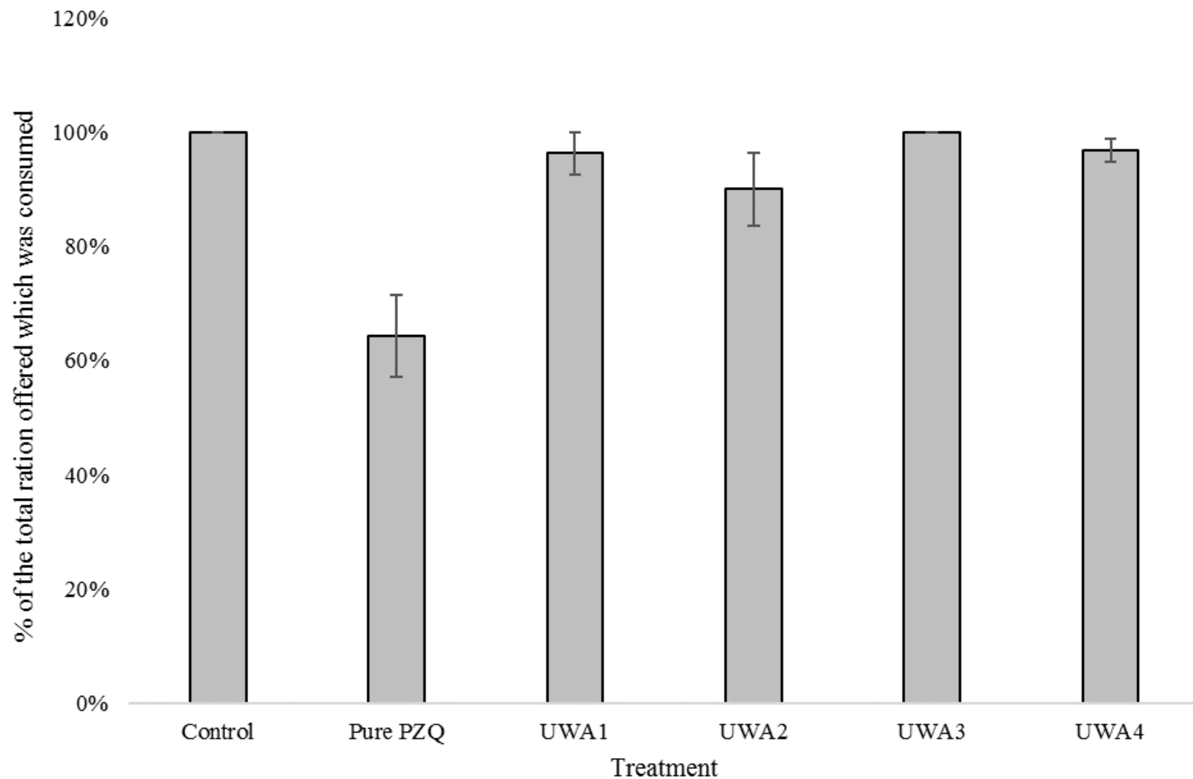


Figure 7: Percentage of the total ration offered which was consumed by 172 gram YTK in Trial 1

Fish offered the pure PZQ diet ate their entire ration on only one occasion (time 2.5 minutes) and this treatment therefore had to be excluded from the analysis of variance (n=1). There was no effect of treatment on the time required to consume the ration in the other five treatments (P = 0.66) (Figure 8) i.e. fish offered the UWA diets consumed their ration as fast as the unmedicated control.

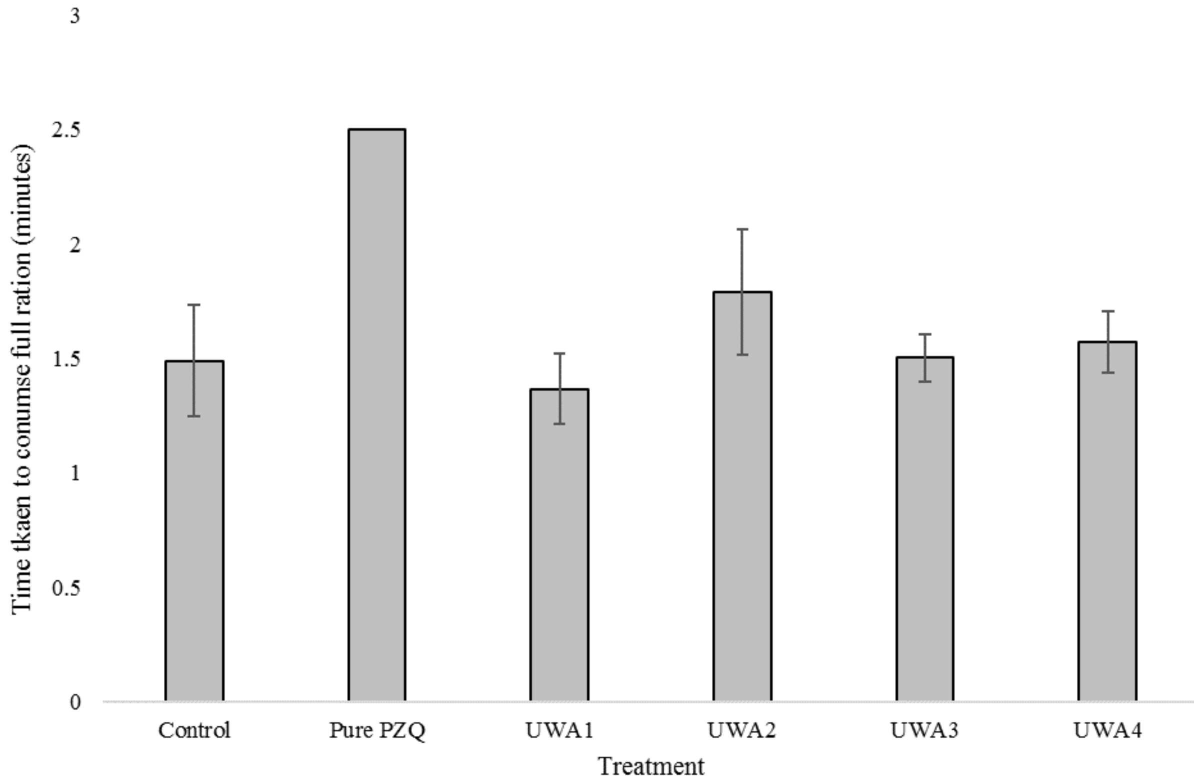


Figure 8: Time taken to consume the ration by YTK in Trial 1 on those occasions when the full ration was consumed.

These data clearly demonstrate that these beads are highly palatable and that the taste/smell of PZQ is very effectively masked. In all UWA-fed tanks, however, subsequent dissection of the fish revealed undigested beads throughout the length of the GI tract (stomach, foregut, midgut and hindgut) (Figure 9). Therefore, despite excellent palatabliity it was clear from these results that the beads were not being digested by the fish. On this basis, UWA re-formulated the beads to be more digestible.

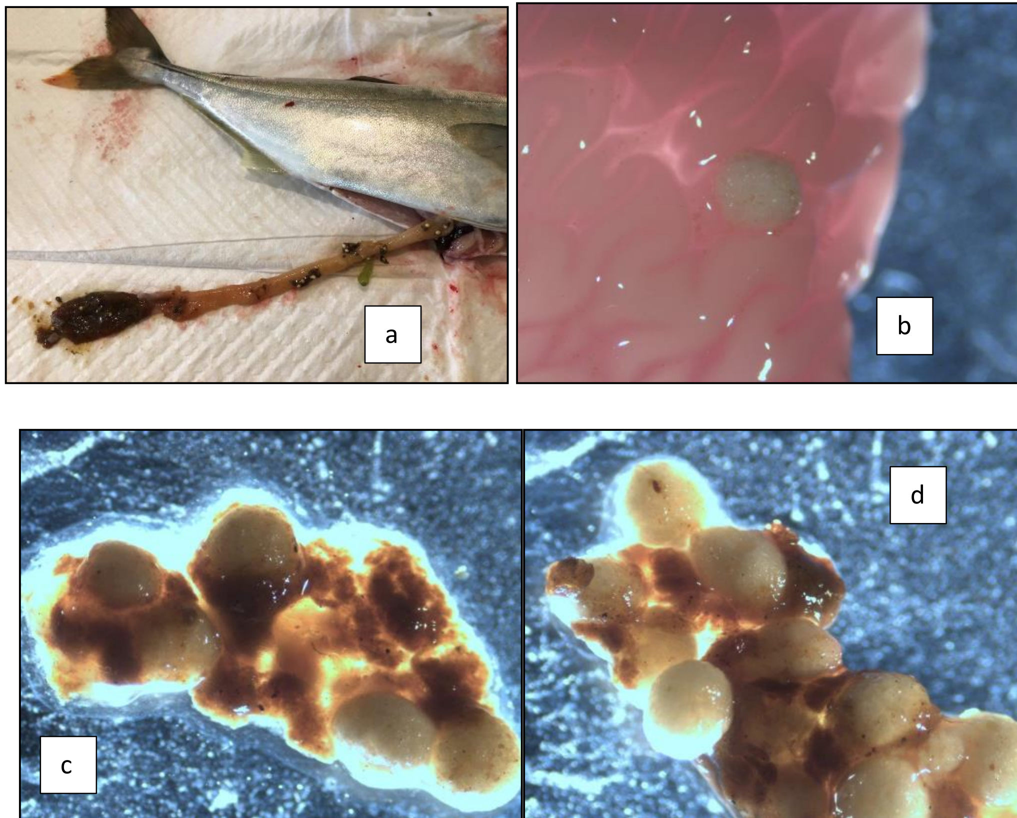


Figure 9: a) Dissected digestive tract showing undigested beads in all sections of YTK in Trial 1. b) undigested beads in the stomach, c) midgut and d) hindgut.

Trial 2

The beads tested in Trial 2 had a higher loading rate than those formulated for Trial 1, which is advantageous, but were larger in size which is likely a disadvantage, particularly in terms of diet manufacture. Laboratory testing at UWA demonstrated that the beads dissolved in acidic media, suggesting they should be more digestible than those tested in Trial 1.

Fish ate 82% of the unmedicated control diet, significantly more than all other treatments ($P < 0.0001$). When the control treatment was excluded from the analysis of variance, those fish in the UWA5 treatment consumed significantly more food (43%) than those offered the UWA9 diet (13%) ($P = 0.03$). Time taken to consume the ration was not analysed because the full ration was never eaten by the fish in any of the medicated treatments.

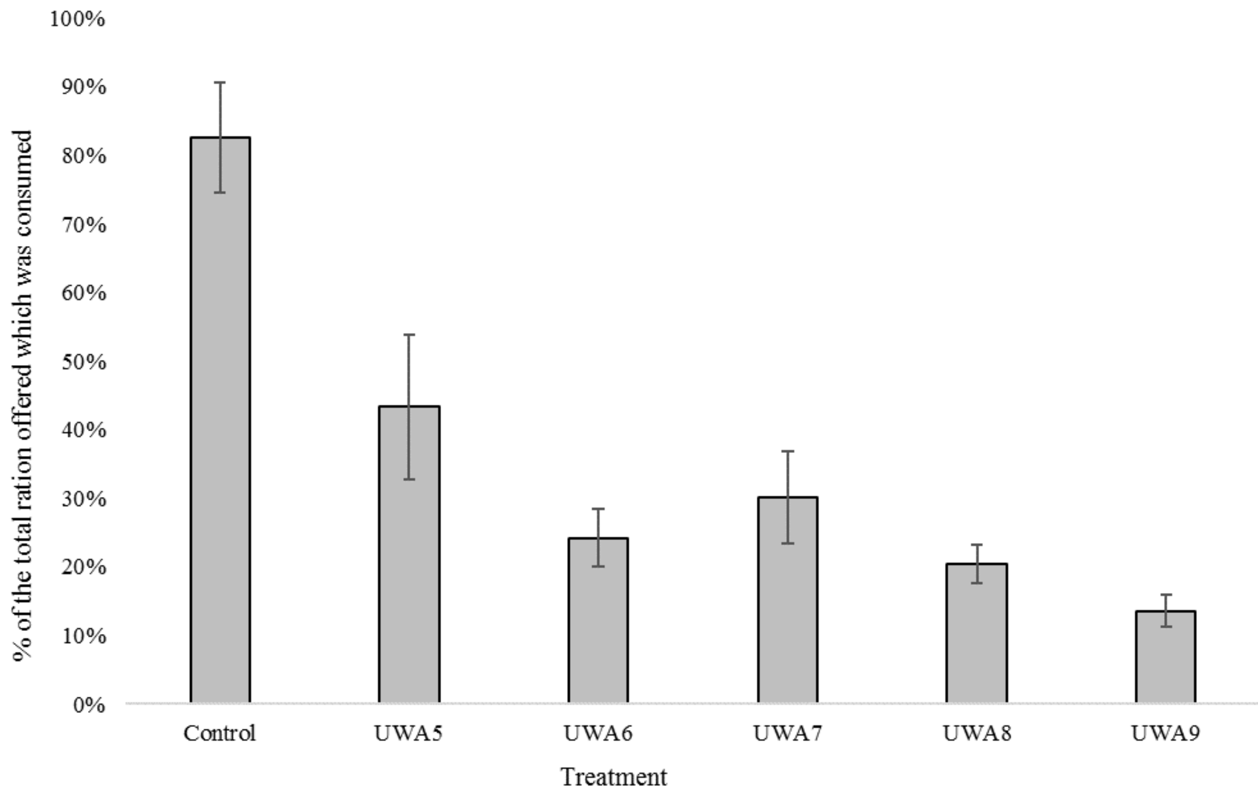


Figure 10: Percentage of the total ration offered which was consumed by 720 gram YTK in Trial 2.

On the basis that fish ate more of UWA5 diet, these fish were selected to check the digestibility of the pellets. Digestibility was assessed by intubating three fish with 14 grams of the diet containing these beads. Two fish were dissected after 11 hours and the third after 15 hours. Photos of the digestive tract from the three fish are shown in Figure 11, Figure 12 and Figure 13. In the two fish dissected after 11 hours, digested feed was found in all sections of the stomach and gut, with undigested beads found only in the stomach. Some beads in the stomach appeared to be trapped in the stomach folds, which may be advantageous in terms of gut retention. The lack of beads in the foregut, midgut and hindgut suggested they had been fully digested, however we could not discount the possibility that they had not travelled into the intestine yet (particularly if they were trapped in the stomach folds). In the fish dissected after 15 hours, only 1 intact bead was found in the stomach and evidence was found of digestion of beads in the other gut sections, supporting the laboratory findings that the beads break down and presumably release their PZQ in acidic media. The agglomeration of beads in the hindgut of Fish#1 after 15 hours may suggest that these beads were not fully digested.

These data suggested that the digestibility issues encountered with the beads in Trial 1 had been overcome, but at the expense of palatability, however we could not be certain that the palatability issues were the result of the beads, or the size of the fish, as the fish in this trial were larger (average 720 g) than the previous trial (average 172 g) and our previous research has suggested that larger fish are more susceptible to the taste/smell of PZQ than small fish (Partridge et al., 2014).

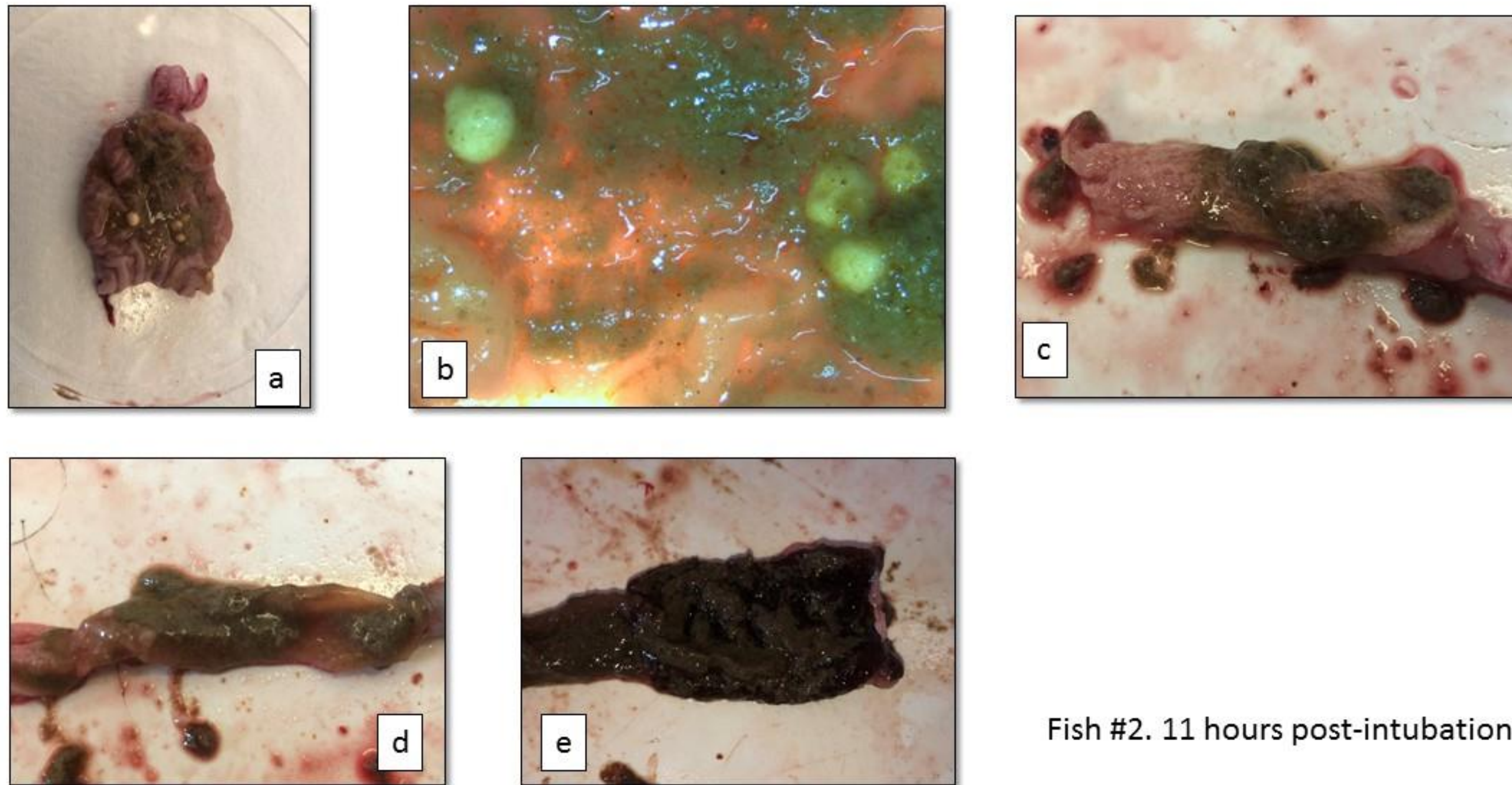


Figure 11: Digestive tract of fish #2 11 hours post-intubation from Trial 2. Undigested beads in a) stomach and b) under the microscope. C) foregut; no evidence of beads d) midgut; no evidence of beads e) hindgut; no evidence of beads

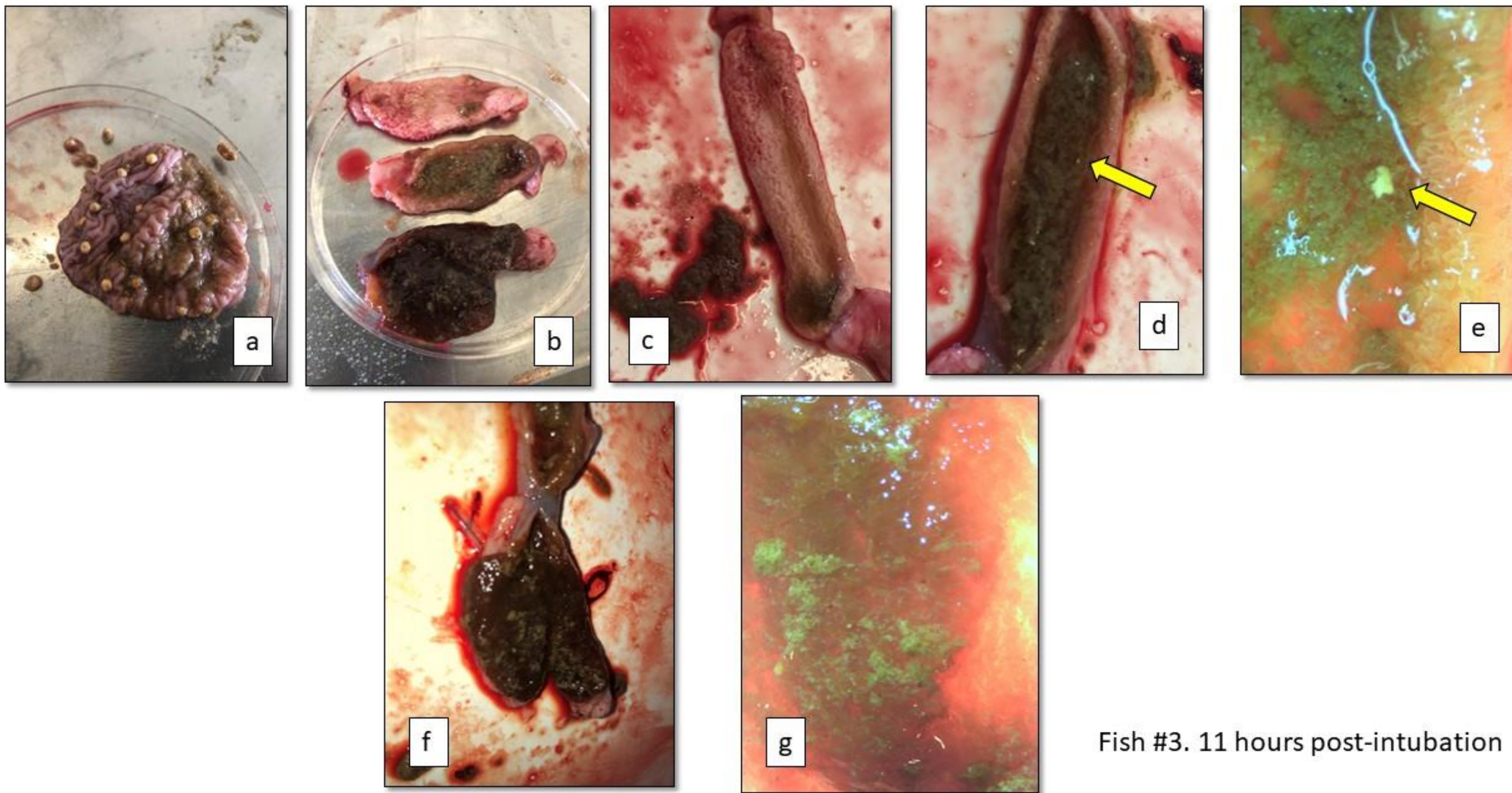


Figure 12: Dissected digestive tract of YTK (fish #3) 11 hours post-intubation from Trial 2. Undigested beads in a) stomach; b) sections of foregut, midgut and hindgut (top to bottom); c) foregut; d) mid-gut showing a white fleck (yellow arrow); e) white fleck under the microscope; f) hindgut g) hindgut under microscope.

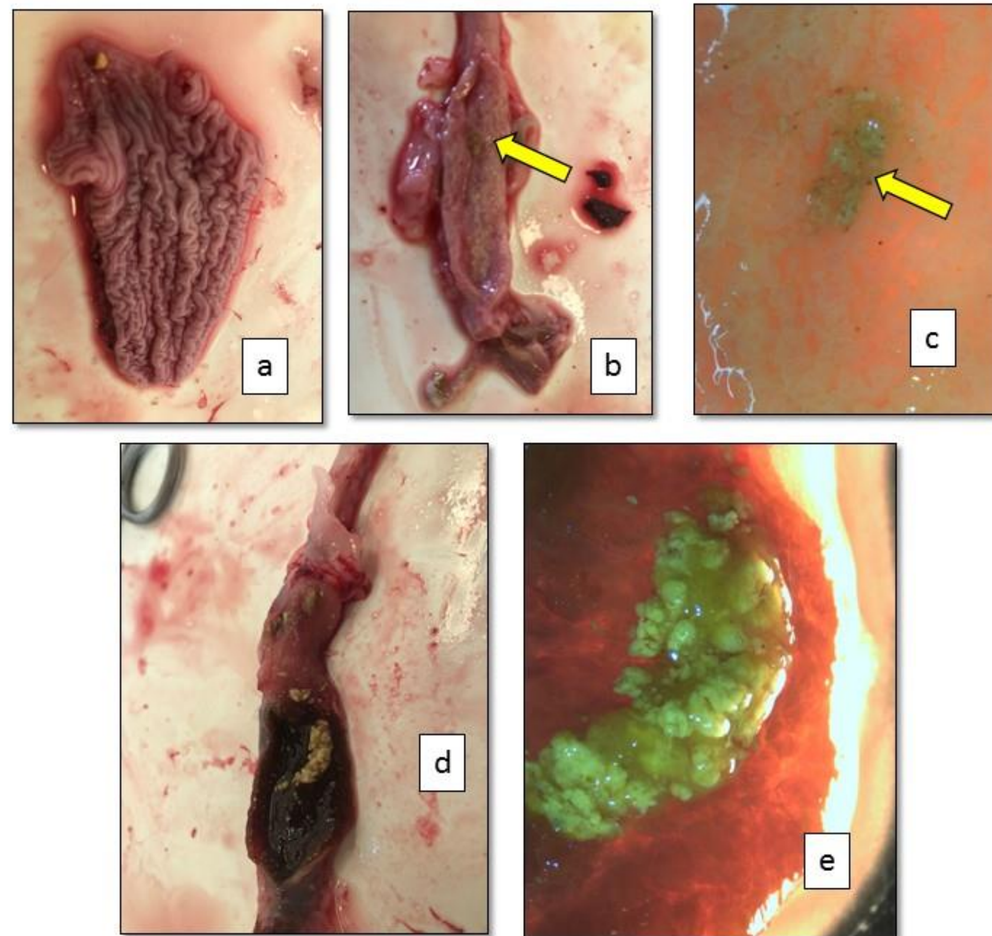


Figure 13: Dissected digestive tract of YTK (fish #1) 15 hours post-intubation in Trial 2. Only 1 bead in the in a) stomach; b) foregut showing partially digested bead (yellow arrow); c) as for (b) but under microscope; d) partially digested beads in hindgut; e) as for (d) but under microscope.

Trial 3

This trial compared palatability and digestibility of beads between fish with average sizes of 175 g (small) and 2000 g (large) using beads with a similar but refined formulation to those tested in Trial 2.

Two-way ANOVA revealed there to be no effect of fish size (P = 0.77) or diet (P = 0.07) on ingestion. Fish fed the UWA E treatment ate on average 75% of their offered ration compared to fish fed the UWA A treatment which only ate on average 68% of the ration. Fish in the unmedicated control treatment consumed 90% of the ration (pooled across small and large fish). These results were contrary to our hypothesis that larger fish are more susceptible to the taste and/or smell of praziquantel. The small control fish ate their entire ration of unmedicated feed, but only ate 60% and 70% of UWA A and UWA E treatment rations, respectively (Figure 14).

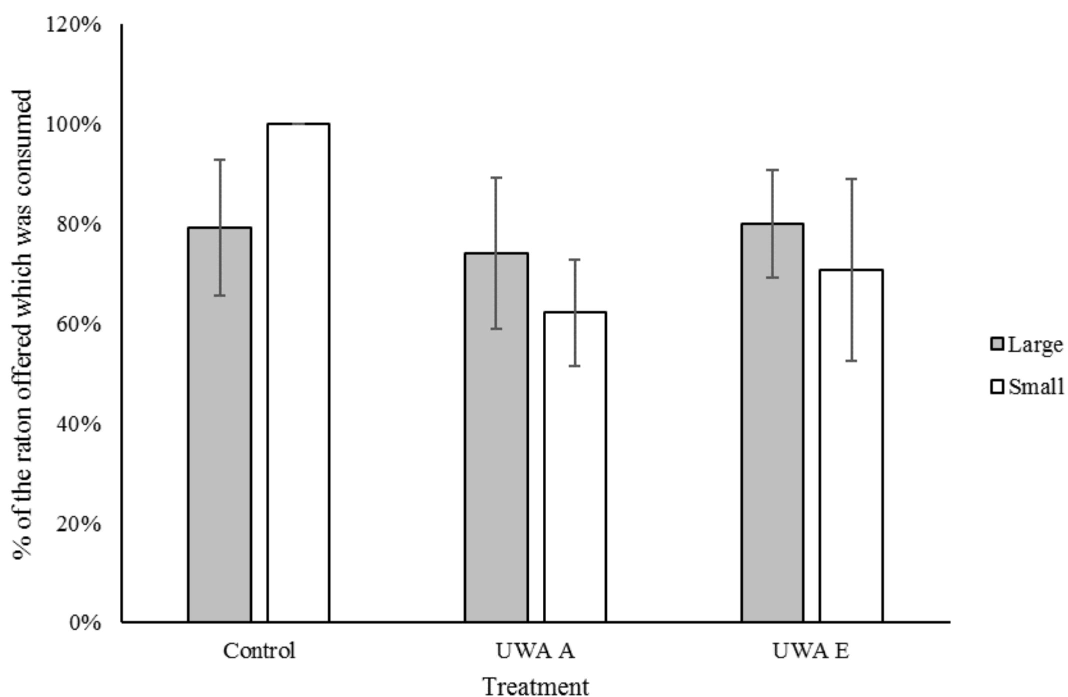


Figure 14: Percentage of the total ration offered which was consumed by 175 gram (small) and 2000 gram (large) YTK in Trial 3.

Time taken to consume a full ration was significantly affected by treatment (P < 0.0001) but not fish size (P = 0.27) (Figure 15). On those occasions when the fish ate the entire medicated ration, the time to do so was about 3 minutes. Fish offered the unmedicated control treatment consumed their ration in about 1.5 minutes.

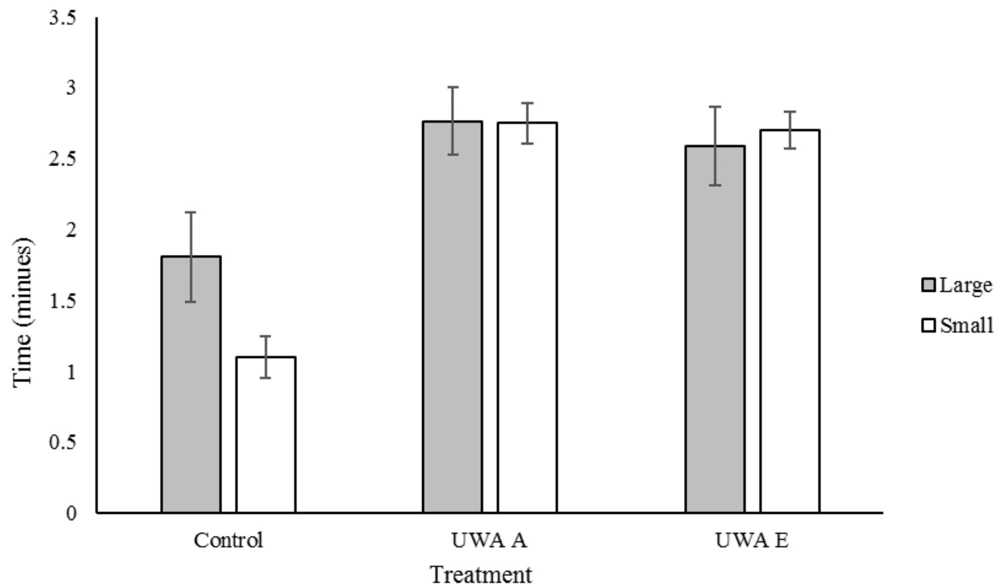


Figure 15: Time taken to consume the full ration by 175 gram (small) and 2 kg (large) YTK in Trial 3.

There was no evidence of beads found in the digestive tract in either small or large fish fed the UWA A treament. Undigested and partly digested beads of the UWA E treament were found through the entire length of the tract in the large fish (Figure 16).

Results of this trial support the findings from Trial 2 that this bead formulation is not consumed as well as those beads tested in Trial 1 but that digestiblity (at least of UWA A) is good.

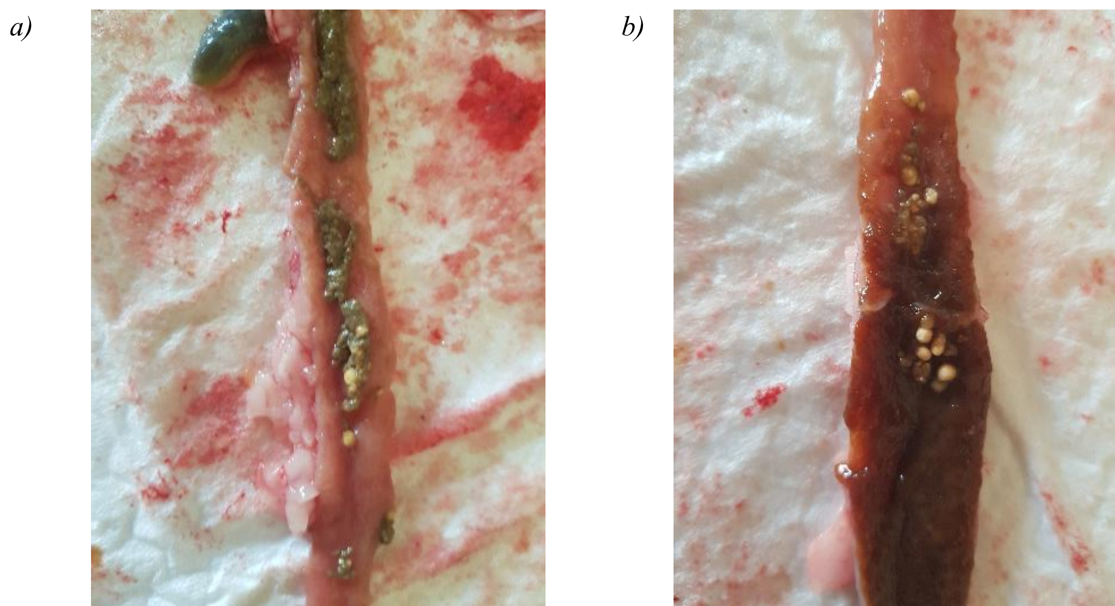


Figure 16: Dissected digestive tract showing undigested beads in the a) midgut and b) hindgut of a large YTK fed UWA E from Trial 3.

Trial 4

As there was no evidence of larger fish being more susceptible to the taste/smell of praziquantel in UWA formulations, only small fish were used in Trial 4, which tested three newly refined formulations. Fish ate ≥99% of all treatments diets (Figure 17), with no difference between treatments (P = 0.28). Whilst the fish ate their full ration at each feeding, the time taken to consume the ration was significantly different between treatments ($P = 0.02$). There was no difference in the amount of time required for fish fed diets containing UWA B (1.15 ± 0.14 minutes) and UWA C (1.16 ± 0.14 minutes) to eat their ration compared with the control (0.64 ± 0.07 minutes), but those fed the diets containing UWA D took significantly longer than the control to the consume the ration (1.42 ± 0.04 minutes)(Figure 18).

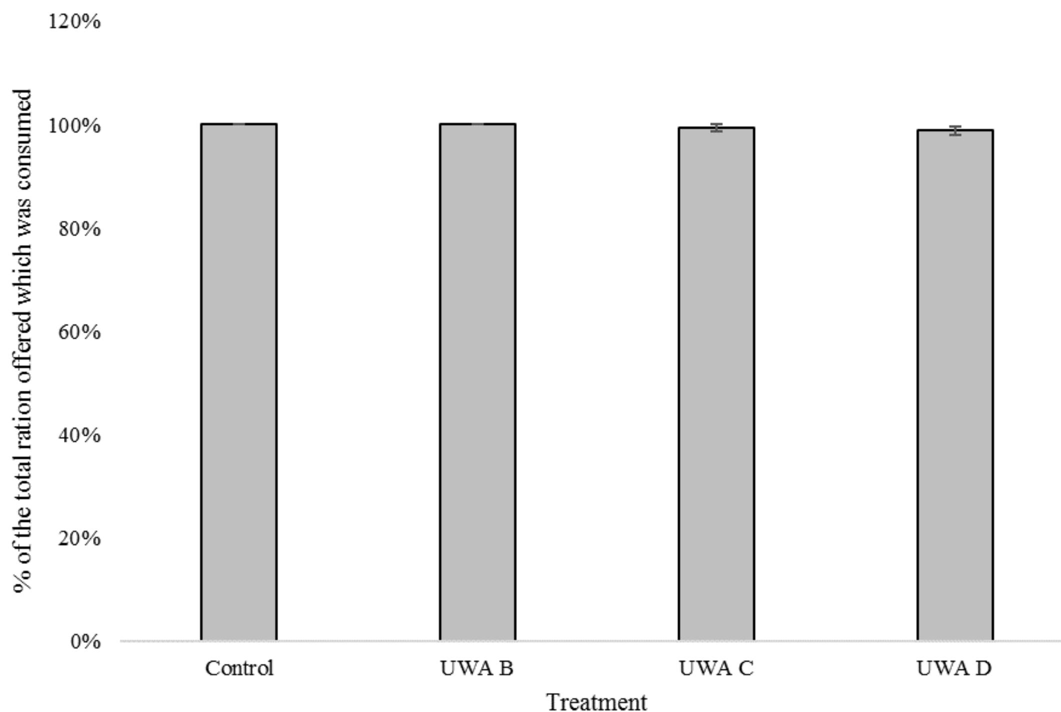


Figure 17: Percentage of the total ration offered which was consumed by 260 gram YTK in Trial 4.

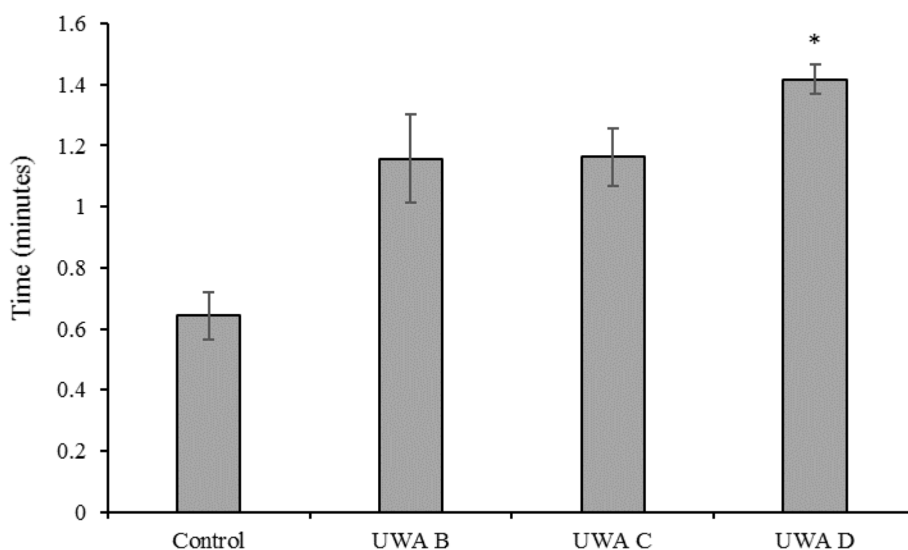
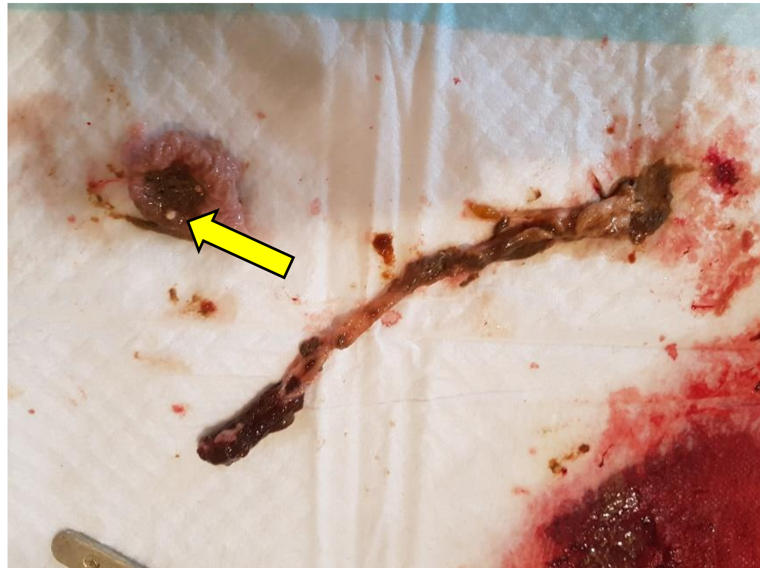


Figure 18: Time taken to consume the ration by 260 gram YTK on those occasions when the full ration was consumed in Trial 4. Symbol asterisks indicates significant differences between control and treatment.

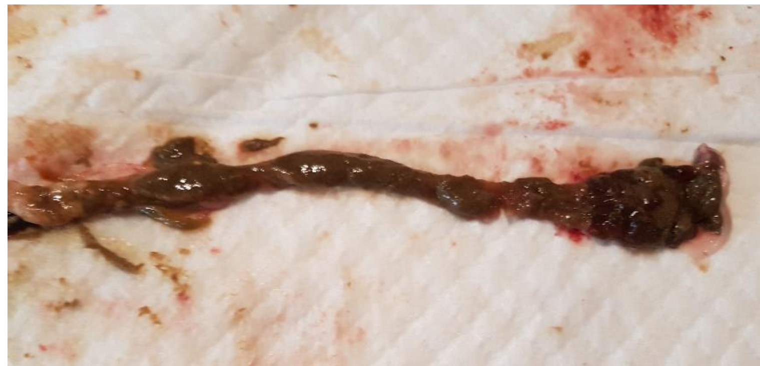
Prior to feeding on Day 5, one fish per treatment was dissected to determine if there were beads remaining in the digestive tract from the previous days feeding. Apart from a single UWA D bead in the stomach, there was no digesta or evidence of beads in the digestive tracts of fish from all treatments. All fish were fed their respective treatment diets and two fish per treatment were dissected 3 and 6 hours post-feeding. Photos of the digestive tract from three treatment fish are shown in Figure 19. By 3 hours post feeding, the feed had moved through the entire length of the fish gut. There were whole beads in the stomach but no evidence of the beads in any section of the tract for all treatments. Similar findings were made 6 hours post feeding.

The results of this final trial demonstrate that a formulation has been prepared that is both highly palatable and digestible.

a)



b)



c)

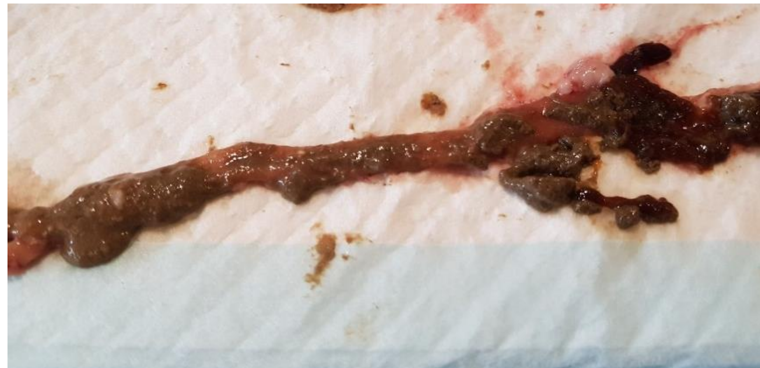


Figure 19: Digestive tract of fish 3 hours post-feeding from Trial 3. a) Undigested beads in stomach (yellow arrow) and no evidence of beads in the gut of fish fed UWA B beads, b) no evidence of beads in gut of fish fed UWA C beads, and c) no evidence of beads in gut of fish fed UWA D beads.

Trial 5

Food intake was significantly lower in fish fed pure PZQ (17 ± 4%) than all other treatments, which did not differ significantly from each other (Figure 20). Fish fed the non-medicated control diet consumed 79 ± 6% of the ration and the two UWA treatments consumed about 62% of the ration offered. None of the diets were consumed at 100% and we believe this was the result of the fish still acclimating to the tank conditions after having been moved from the seacage. Feed intake recorded each day (Figure 21) shows that all tanks fed poorly on the second day and that feed intake subsequently increased in all treatments over the following days, with the exception of the pure PZQ which remained low. The fact that the intake of UWA treatment diets increased over time, similarly to the control diet, is further evidence that the diets are highly palatable, as intake in pure PZQ diets typically remains low over time.

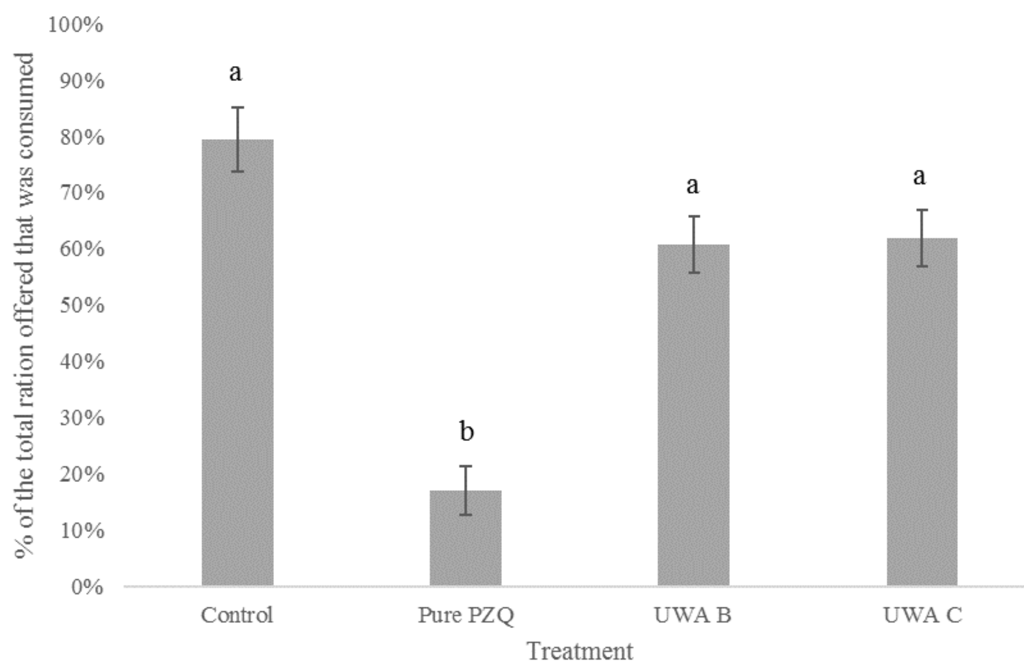


Figure 20: Percentage of the total ration offered which was consumed by 1600 gram YTK in Trial 5.

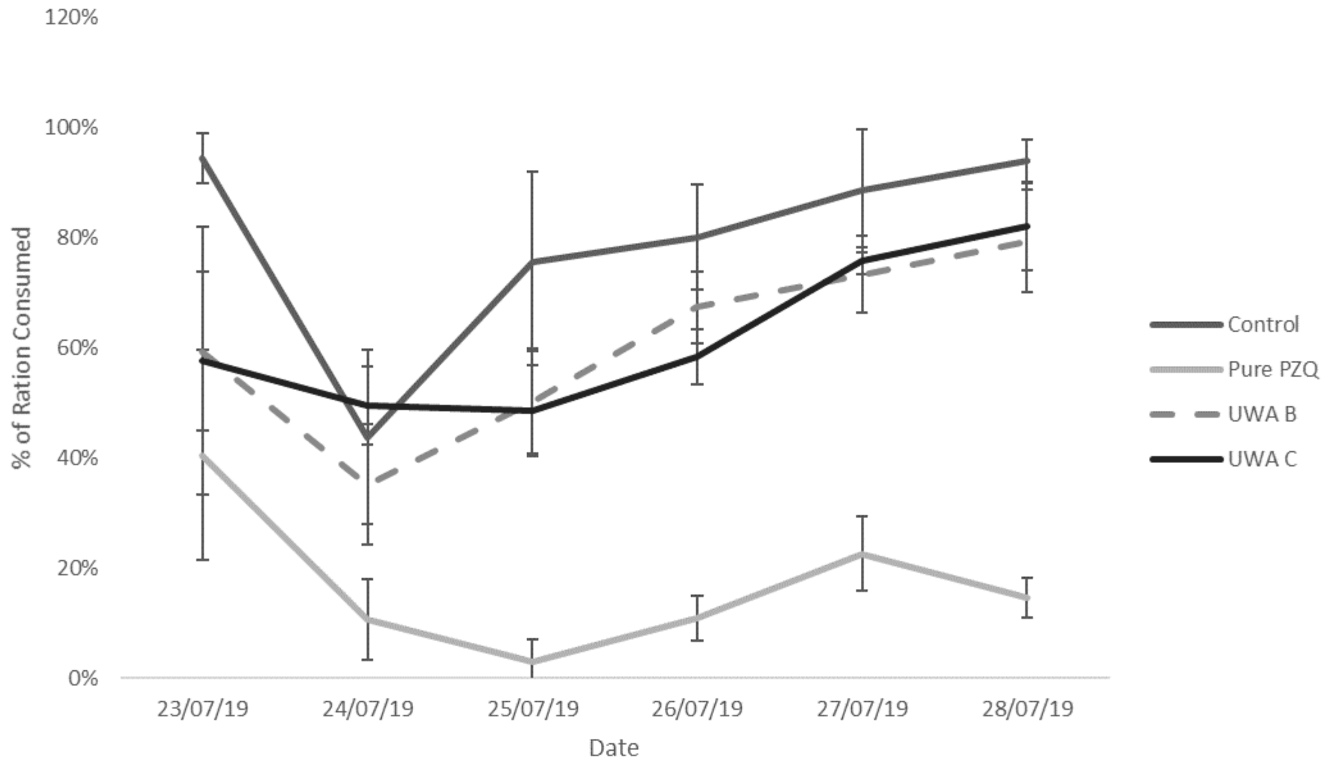


Figure 21: Ingestion of diets by day over the 6 day experimental period of Trial 5.

Based on the actual amount of feed consumed, the average daily dose of PZQ received by fish in each treatment was calculated (Figure 22). Fish fed the UWA medicated diets received a significantly higher average daily dose of PZQ (ca. 75 mg/kg) ($P < 0.001$) than those fed the pure PZQ diet (ca. 21 mg/kg). It is generally considered that fish must receive approximately 50 mg/kg for three consecutive days to eliminate *Zeuxapta* (Partridge et al., 2014). This dose was easily achieved in the UWA treatment but not in the pure PZQ treatment.

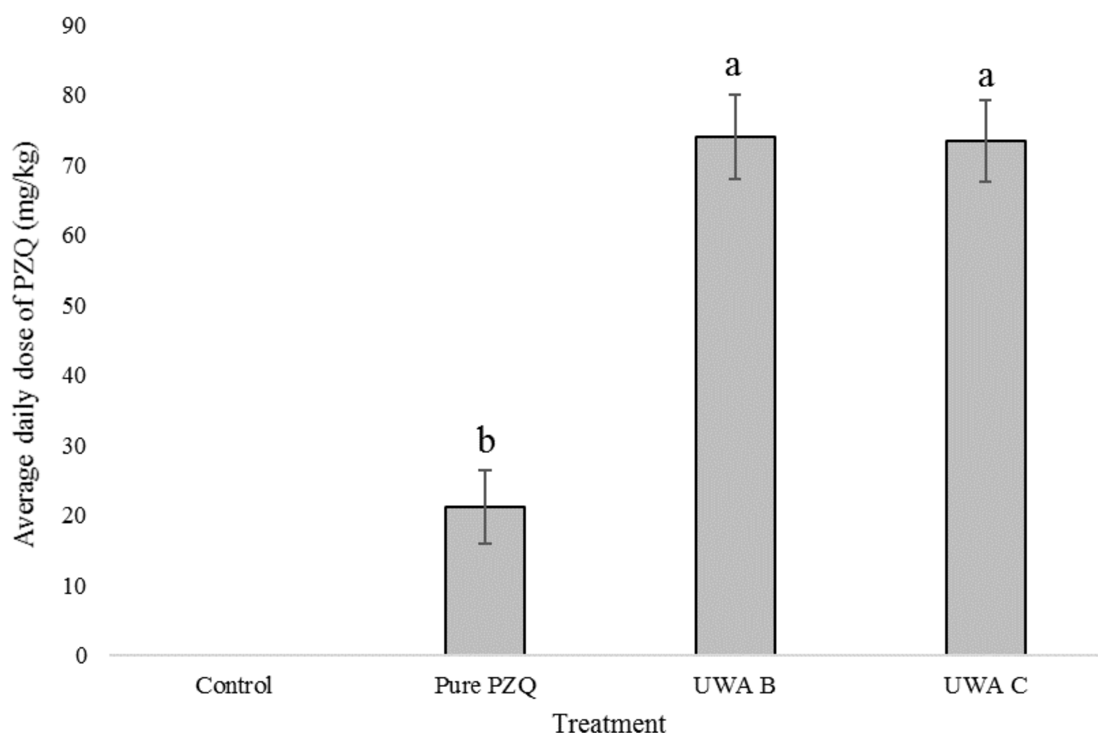


Figure 22: Average daily of PZQ intake (mg/kg BW) by YTK in the four treatments.

Fish fed the unmedicated control diet had an average of 55 ± 13 *Zeuxapta* flukes per fish at the end of the trial. There was a significant effect of diet on the percentage reduction in fluke numbers in the three medicated treatments relative to the control ($P < 0.001$). Fish fed the UWA B and UWA C treatments had reductions of 93 ± 2% and 94 ± 3%, respectively, both significantly higher than the pure PZQ control, which experienced a 73 ± 4% reduction (Figure 23).

These data demonstrate that UWA formulations B and C are both palatable, digestible and efficacious against *Zeuxapta* infections. Investigations are currently underway to determine the commercialisation potential of this new formulation in collaboration with UWA.

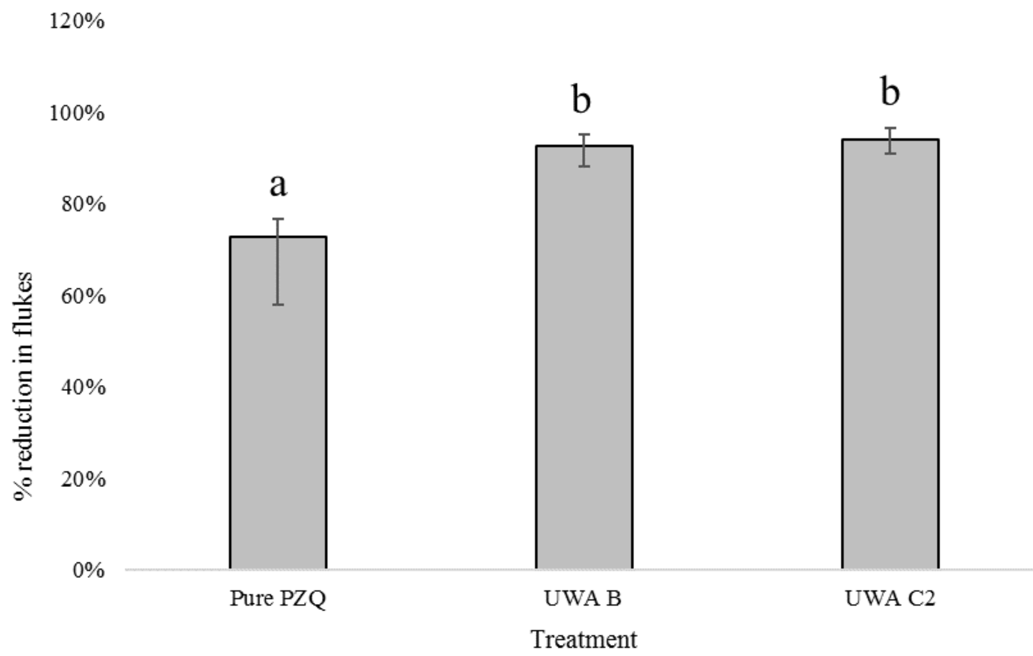


Figure 23: Percentage (%) reduction in *Zeuxapta* flukes on YTK fed three diets containing praziquantel relative to the non-medicated control diet.

3. *Quantify the benefits of an autogenous vaccine against* Photobacterium damselae *subspecies* damselae.

Results of the first trial conducted at the DPIRD Geraldton Annex showed a positive response in vaccinated fish against both of the antigens in the vaccine, and that this response can be titrated out effectively (Figure 25). There is a wide spread of responses across the vaccinated fish, which is not unusual, but the vaccination significantly (P<0.0001, 2-way ANOVA) increased the specific circulating antibody. The much tighter spread of data around the unvaccinated fish in response to QMA509 may suggest that these fish were naive to this strain, whilst the larger spread of data in the unvaccinated fish against QMA0510 may suggest these fish had been exposed to this strain before. None of the fish suffered from Photobacteriosis during this trial

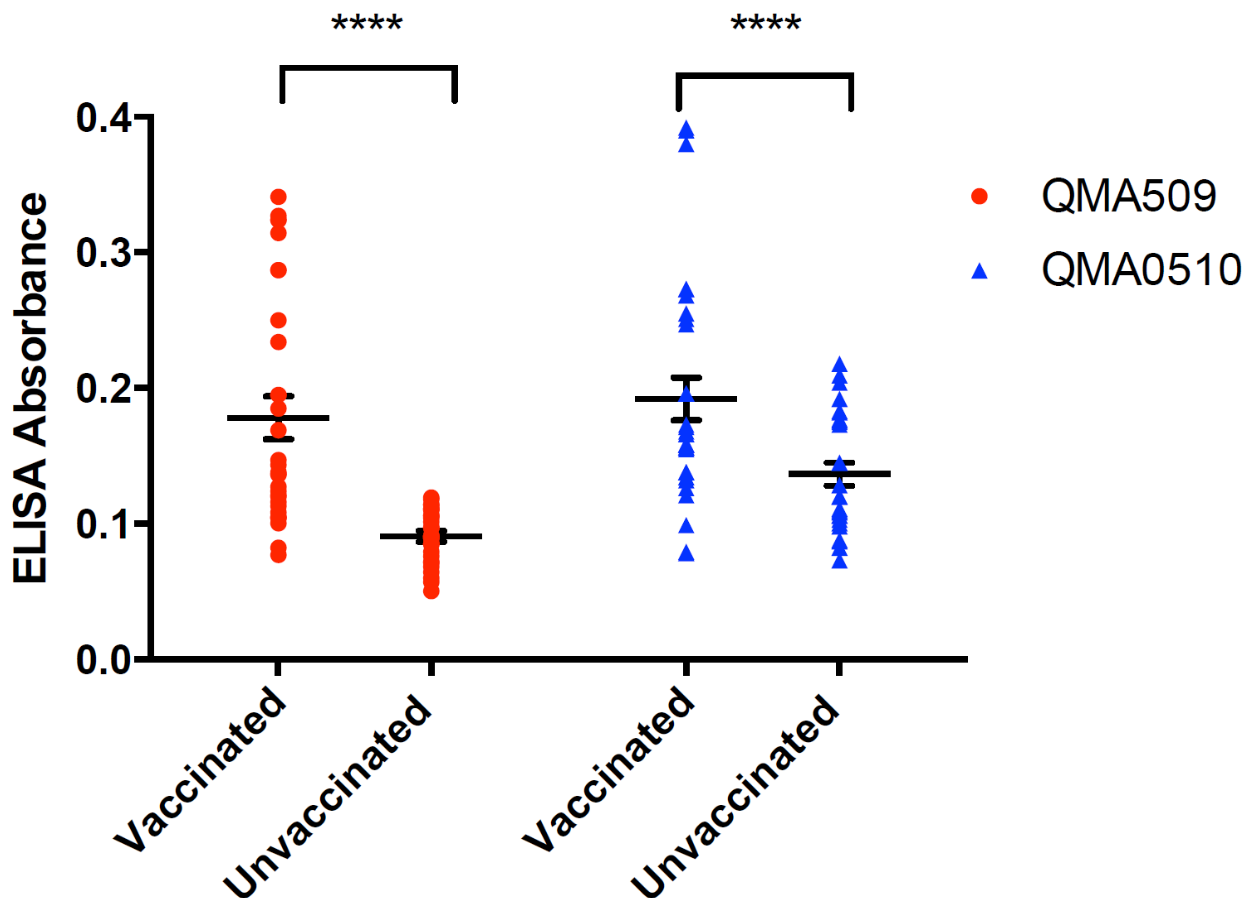


Figure 24: Antibody response of vaccinated and unvaccinated YTK against two strains of *Photobacterium damselae* subspecies *damselae*.

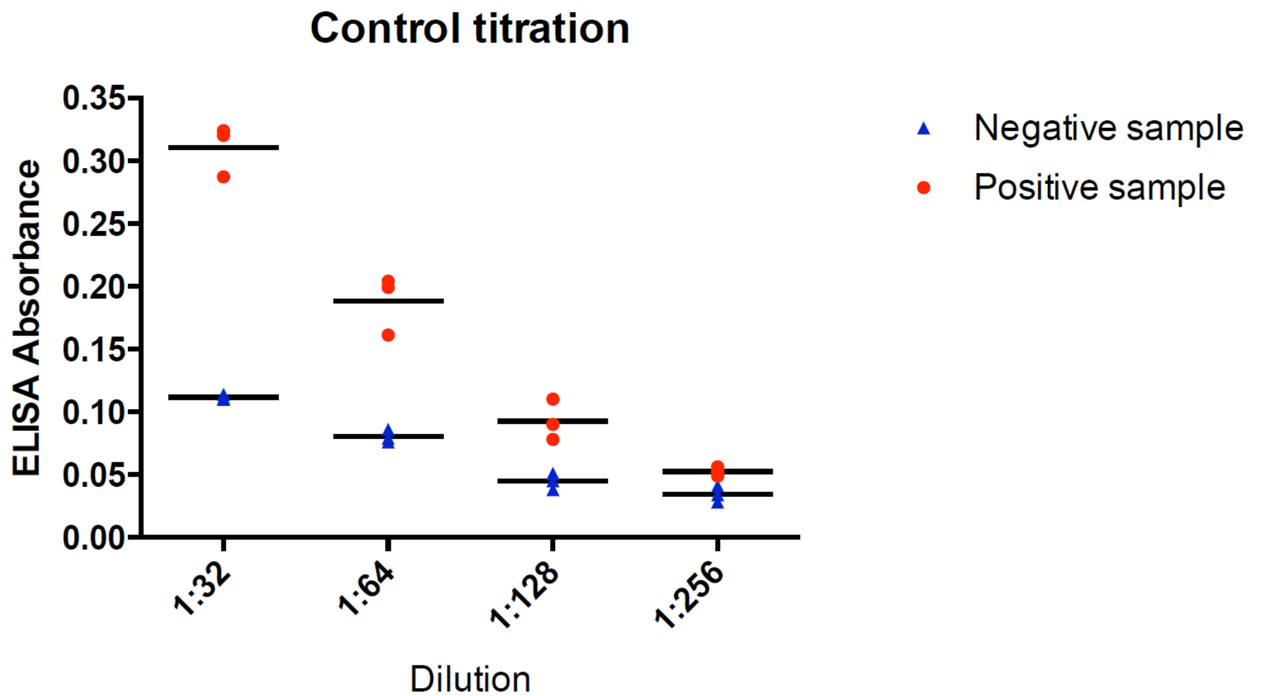


Figure 25: Titration response of antibodies in YTK plasma.

Results of the more comprehensive 'prime-boost' study showed that background (or natural) antibody in the cohort of fish rose steadily over time in the experiment, and this was paralleled in repeat-bled individuals as well as mean across the cohort (Figure 26 A-D). One of the major means of innate defence in fish is the utilisation of natural, non-specific, polymeric IgM (Pilström & Bengtén, 1996) to bind and immobilise invading organisms by cross-linking. In general, total IgM levels in serum increase in fish with size/age (Magnadottir, Gudmundsdottir, Gudmundsdottir, & Helgason, 2009) but there is some variability dependent upon sex (Picchietti et al., 2001) or disease status (Gudmundsdóttir, Magnadóttir, Björnsdóttir, Árnadóttir, & Gudmundsdóttir, 2009), and this is consistent with the variable findings here for YTK, albeit supported by the strongly increasing trend with age (Figure 26 A, B). It is also highly likely that fish were continually re-exposed via natural routes to similar serotypes of Pdd during the course of the trial as these strains have been isolated from fish held in this facility. However, natural exposure is unlikely to induce high levels of serum antibody in the absence of overt infection as only mucosal surfaces will be challenged and this does not elicit specific systemic immunity (Delamare-Deboutteville, Wood, & Barnes, 2006).

The ability of vaccinated kingfish to mount a rapid secondary antibody response was clearly evident in vaccinated fish that were re-exposed to the same mixture of antigens by injection 100 days post primary immunisation and then bled at 107 days (Figure 26). This is consistent with findings in other fish and this rapid secondary response is the primary means of vaccine-induced protection in fish. Unvaccinated fish that were exposed by injection at this time did not mount a significant secondary response to the antigens evidenced by the substantially lower specific serum antibody levels 7 days post immune challenge (Figure

26). This supports our contention above that increase in serum antibody detected through time was a result of age rather than natural exposure, as systemic exposure would elicit priming for a similarly elevated, rapid secondary response to vaccinated fish.

The results of this study form the basis of the current FRDC Project "2018-101 A trivalent vaccine for sustainable Yellowtail Kingfish growout", which will build upon this basic formalin killed bacterin and deliver improved vaccines against Pdd as well as Vh and *Photobacterium damselae* subspecies *piscicida* (Pdp).

4. *Characterisation of Australian strains of* Photobacterium damselae *subspecies* damselae *and* Vibrio harveyi.

Table 5 summaries the results of the phenotypic characteristics of the 16 Pdd isolates. Based on these results, the 16 isolates were categorised into 5 different biotypes or phenotype profiles. All biotypes, were negative for bioluminescence and swarming activity whereas only AS-17-7468#12 exhibited positive activity for Voges-Proskauer, resulting in this strain being designated a unique biotype (#4). No isolates grew at 4°C but all grew at both 24 ºC and 37ºC. All biotypes required salt for their growth. Although strains of this pathogen are typically considered to produce green colonies on TCBS medium, it was found that one biotype (#5) produced yellow colonies. This biotype was the only one which was positive for sucrose fermentation. The isolates showed variability for trehalose, MR, ornithine decarboxylase, lysine decarboxylase, arginine decarboxylase, Dnase and vibriostatic disk.

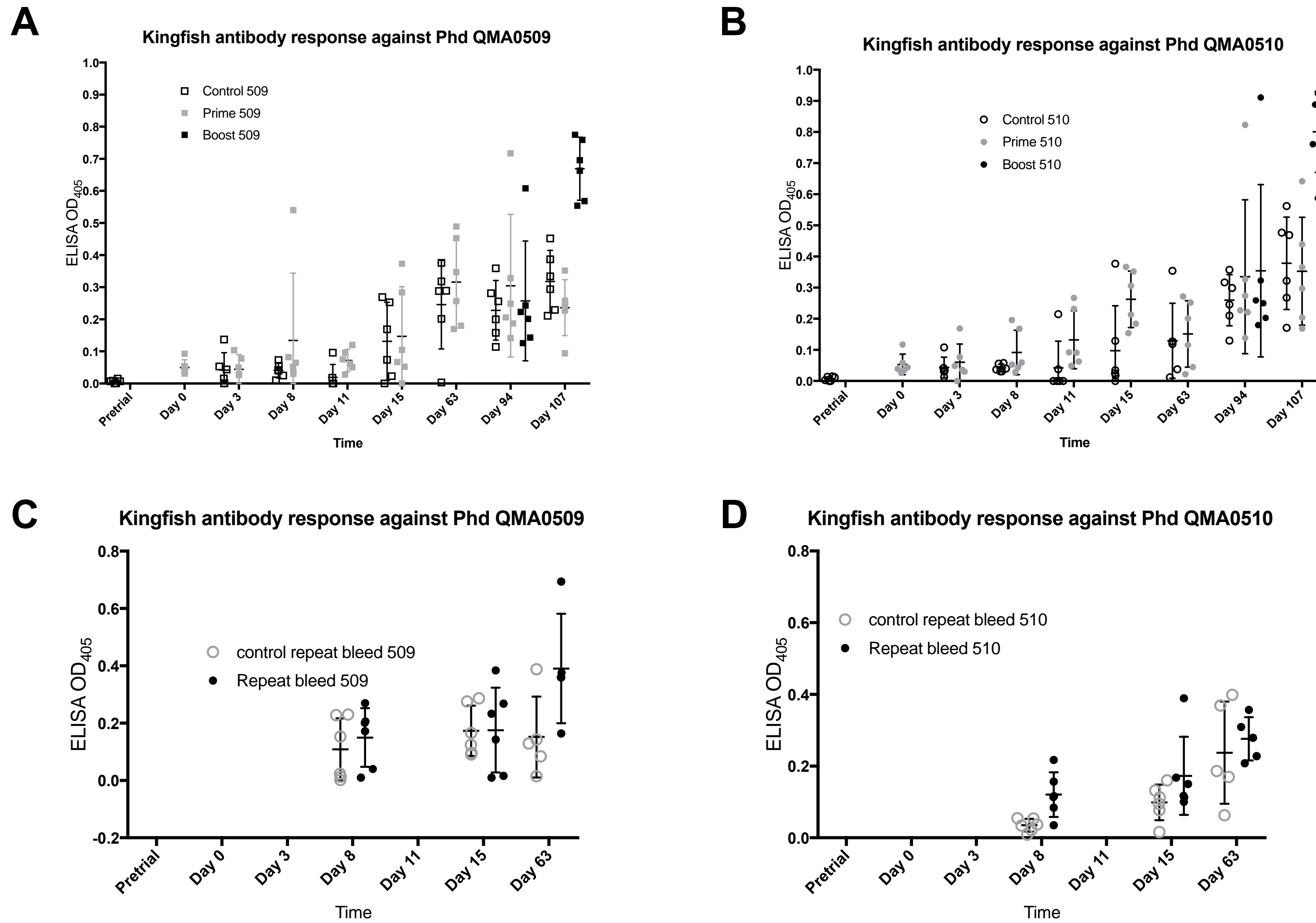


Figure 26: Antibody responses of YTK over time against two strains of *Photobacterium damselae* subspecies *damselae*.

Table 5: Phenotypic characteristics of 16 *Photobacterium damselae* subspecies *damselae* isolates

Characteristics	AS-15-3942#7	AS-15-3942#8	AS-15-3942#9	AS-16-0963#1	AS-16-0963#3	AS-17-6307#26	AS-17-6307#61	AS-17-6307#69	AS-17-7468#8	AS-17-7468#12	AS-18-0902#12	AS-18-0902#17	AS-14-1386#1	AS-16-2454#19	AS-18-2495#3	AS-18-2495#5
Growth on TCBS	Green	Green	Green	Green	Yellow	Green	Green	Green	Green	Green	Yellow	Green	Green	Green	Green	Green
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at temperature																
4°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at salinity																
0% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bioluminescence	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
Methyl Red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Ornithine Decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine Decarboxylase	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-
Arginine Decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sensitivity to																
0/129 150 µg	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
0/129 10 µg	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S
Dnase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chitinase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biotype	**1**	**1**	**1**	**2**	**5**	**1**	**3**	**3**	**3**	**4**	**5**	**3**	**1**	**3**	**3**	**3**

The haemolytic activity of these 16 isolates on horse and YTK blood is shown in Figure 27. Five of the 16 isolates showed strong haemolytic activity (>10 mm haemolytic zone diameter on both horse and YTK blood) and the rest were considered as weakly haemolytic (<10 mm haemolytic zone diameter). Two of the five highly haemolytic isolates originated from snapper and two of the remaining three highly haemolytic isolates from YTK originated from the same diagnostic case. In all five isolates that displayed strong haemolytic activity, the haemolytic zone diameter on YTK blood was significantly larger than on horse blood. The relationship between haemolytic zone diameter on horse and YTK blood was not as clear for weakly haemolytic strains, with some of these strains having a larger haemolytic zone on horse blood. Interestingly, two isolates, AS-18-0902#12 and AS-18-0902#18 which were deemed non-haemolytic on horse blood had a haemolytic zone diameter >8 mm on YTK blood. As noted in Section 3, strains used in the commercial vaccine were deemed to be high and low virulence based on previous measurements of their haemolytic zone on horse blood. These two strains are AS-15-3942 #7 and #8 in Figure 28 below, which have haemolytic zone diameters of 6.5 and 4.2 mm on horse blood, respectively. Whilst one strain is high relative to the other, both are low/weak compared with those which have haemolytic zone diameters >10 mm.

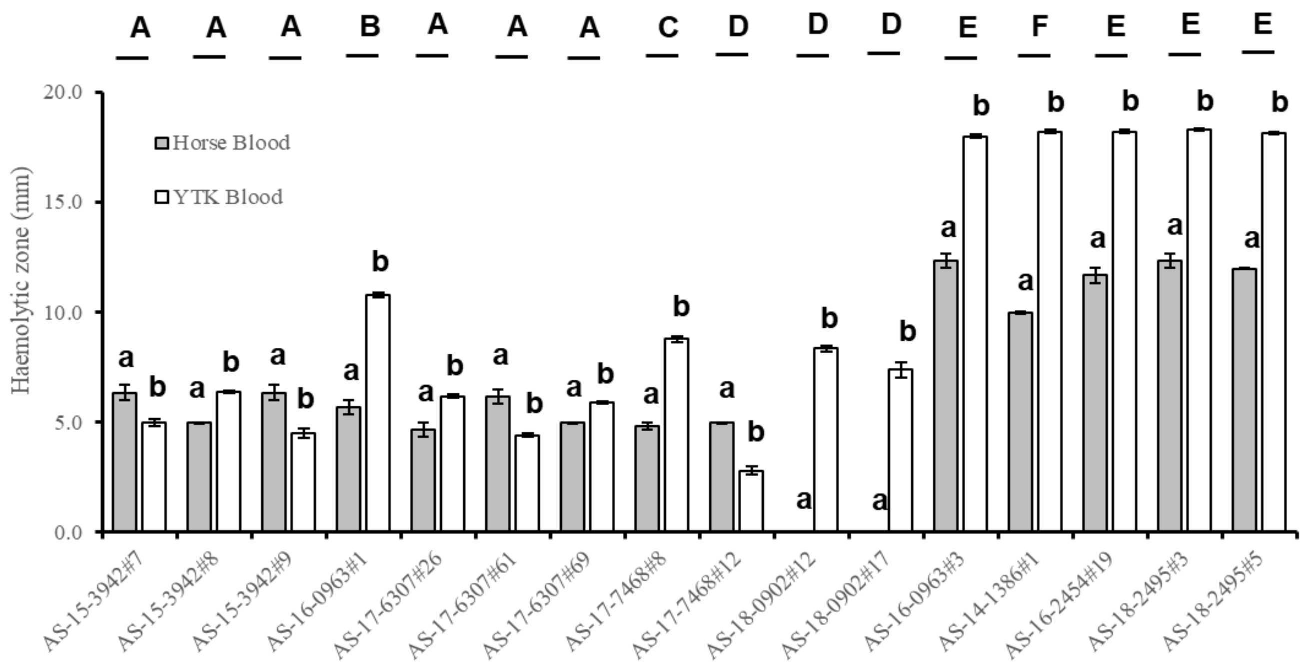


Figure 27: Haemolytic zones produced by different *Photobacterium damselae* subspecies *damselae* isolates on YTK blood or horse blood plates. Different lowercase letters (a, b) denote significant differences between haemolytic zones on horse and YTK blood plates in the same isolates. Bars holding different uppercase letters (A, B, C, D, E, F) among different isolates are significantly different.

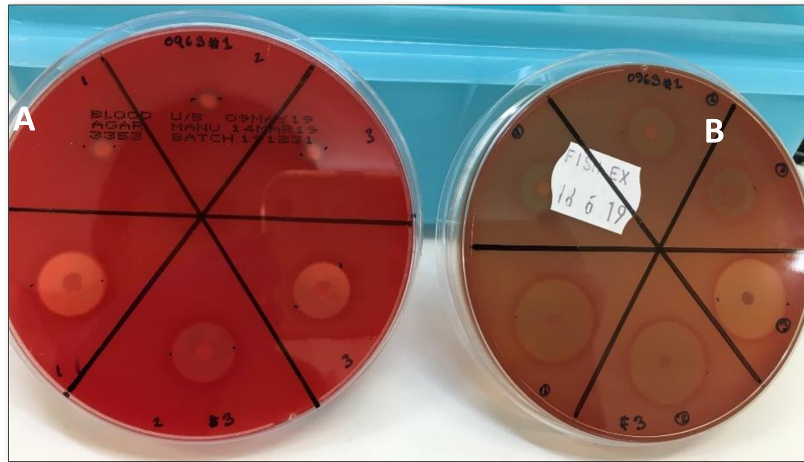


Figure 28: Haemolytic zones produced by *Photobacterium damselae* subspecies *damselae* isolates on horse blood plate (A) and YTK blood plate (B)

PCR amplification with specific virulence gene primers revealed that all five isolates with a strong haemolytic zone (= strong beta haemolysis SBH) harboured the pPHDD1 plasmid and its encoded dly and hlyApl genes (Table 6). Plasmid extraction following gel electrophoresis was performed to confirm the presence of the plasmid. The pPHDD1 and encoded dly amplification products of five strong haemolytic isolates were sequenced and compared with the deposited sequences in NCBI. All showed an almost 100% similarity with the deposited sequence, FN597600. Chromosomal haemolysin genes, hlyAch and PlpV were present in all 16 isolates. These data suggest that the strong haemolytic activity by the five plasmid bearing isolates is due to the presence of the pPHDD1 plasmid and its associated dly and hlyAch genes which are responsible for the production of the toxins damelysin and haemolysin. There was no correlation between the presence of the pPHDD1 plasmid and biotype.

Table 6: Results of the PCR screening for gene markers of 16 isolates of *Photobacterium damselae* subsp. *damselae* (Pdd).

Pdd isolates	Haemolytic Zone		*pPHDD1*	*dly*	*hlyApl*	*hlyAch*	*PlpV*	Bio type
	Horse Blood	YTK Blood						
AS-15-3942#8	**WBH**	**WBH**	-	-	-	+	+	**1**
AS-15-3942#9	**WBH**	**WBH**	-	-	-	+	+	**1**
AS-15-3942#7	**WBH**	**WBH**	-	-	-	+	+	**1**
AS-16-0963#1	**WBH**	**WBH**	-	-	-	+	+	**2**
AS-16-0963#3	**SBH**	**SBH**	+	+	+	+	+	**5**
AS-17-6307#26	**WBH**	**WBH**	-	-	-	+	+	**1**
AS-17-6307#61	**WBH**	**WBH**	-	-	-	+	+	**3**
AS-17-6307#69	**WBH**	**WBH**	-	-	-	+	+	**3**
AS-17-7468#8	**WBH**	**WBH**	-	-	-	+	+	**3**
AS-17-7468#12	**WBH**	**WBH**	-	-	-	+	+	**4**
AS-18-0902#12	**NH**	**WBH**	-	-	-	+	+	**5**
AS-18-0902#17	**NH**	**WBH**	-	-	-	+	+	**3**
AS-14-1386#1	**SBH**	**SBH**	+	+	+	+	+	**1**
AS-16-2454#19	**SBH**	**SBH**	+	+	+	+	+	**3**
AS-18-2495#3	**SBH**	**SBH**	+	+	+	+	+	**3**
AS-18-2495#5	**SBH**	**SBH**	+	+	+	+	+	**3**

SBH= Strong beta haemolysis; WBH= Weak beta haemolysis

Challenge with 10^7 CFU/fish of the plasmid bearing isolate AS-16-0963#3 resulted in 100% mortality, compared with ~20% mortality in those challenged with the same dose of non-plasmid bearing isolates (AS-16-0963#1 and AS-15-3942#7($p<0.05$)(Figure 29).

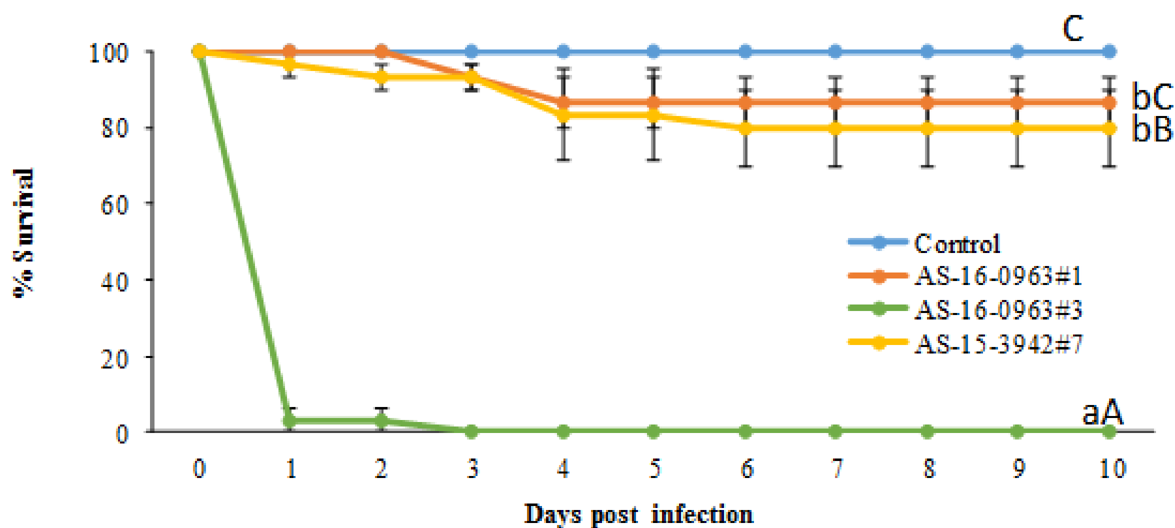


Figure 29: Survival of YTK challenged with 10^7 CFU/fish of three isolates of *Photobacterium damselae* subsp. *damselae* (n=10 fish per dose and per isolates).

Administration of 10^4 CFU/fish resulted in ~50% of fish mortality when challenged with plasmid-bearing AS-16-0963#3. The mortality rates for non-plasmid bearing AS-16-0963#1 and AS-15-3942#7 at the same doses were 3% and 13% respectively.

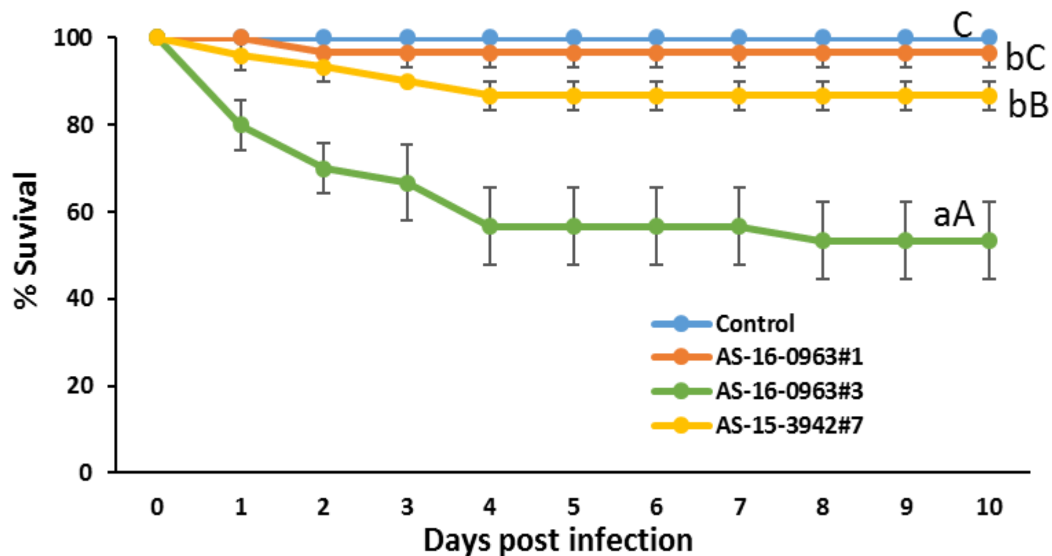


Figure 30: Survival of YTK challenged with 10^7 CFU/fish of three isolates of *Photobacterium damselae* subsp. *damselae* (n=10 fish per dose and per isolates).

Affected fish showed haemorrhage around the mouth and fins and ulcerative lesions (Figure 31). MALDI-TOF and urease test results confirmed the reisolation of *P. damselae* subsp. *damselae* from kidneys of post challenge moribund fish.

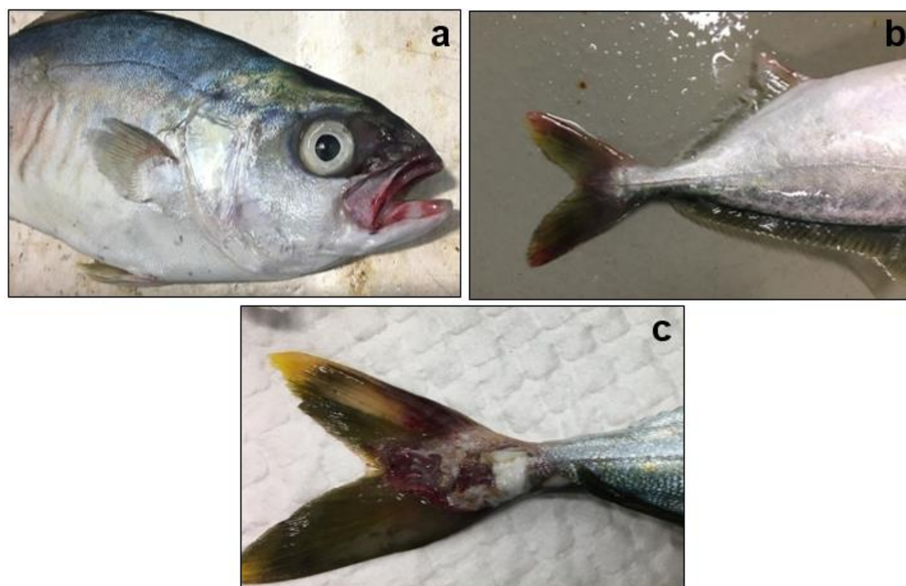


Figure 31: *Photobacterium damselae* subsp. *damselae* injected YTK showing haemorrhages around mouth (a) and fins (b), ulcerative skin lesion near caudal peduncle (c).

3 *Determine whether interactions exist between* Photobacterium damselae *subspecies* damselae *and* Vibrio harveyi *that influence virulence*

3.i. In-vivo *challenge trials.*

The percentage of challenged fish was affected by dose of bacteria ($P < 0.00001$) but not by bacteria type ($P = 0.89$) or their interaction ($P = 0.88$) (Figure 32). In the 10^6 and 10^7 CFU/fish treatments the percentage of fish affected were 42 ± 13% and 89 ± 6%, respectively. These two values were significantly different to each other and to all other treatments. In both cases, the percentage of fish affected by the combination of Pdd and Vhh was equal to that in which the bacteria were applied singularly. This data demonstrates that under these conditions there was no interactive effect of the two bacterial strains that influences their virulence. That such high doses of bacteria (i.e. 10^6 and 10^7 CFU/fish) were required to elicit an effect suggests that neither bacterium is very pathogenic. It was considered that an additional environmental stressor may be required to trigger the virulence genes in these bacteria, however no mortality occurred in any tanks in the 5 days following the low DO stress. This may suggest that the fish had already mounted an effective immune response against the bacteria during the first 10 days and eliminated them, or reduced them to a safe level, or simply that this stressor, or the time applied for the stressor did not trigger the virulence genes.

As previously noted, this trial was conducted prior the strain characterisation and subsequently utilised Pdd strains with low virulence (lacking pPHDD1 plasmid and associated virulence genes).

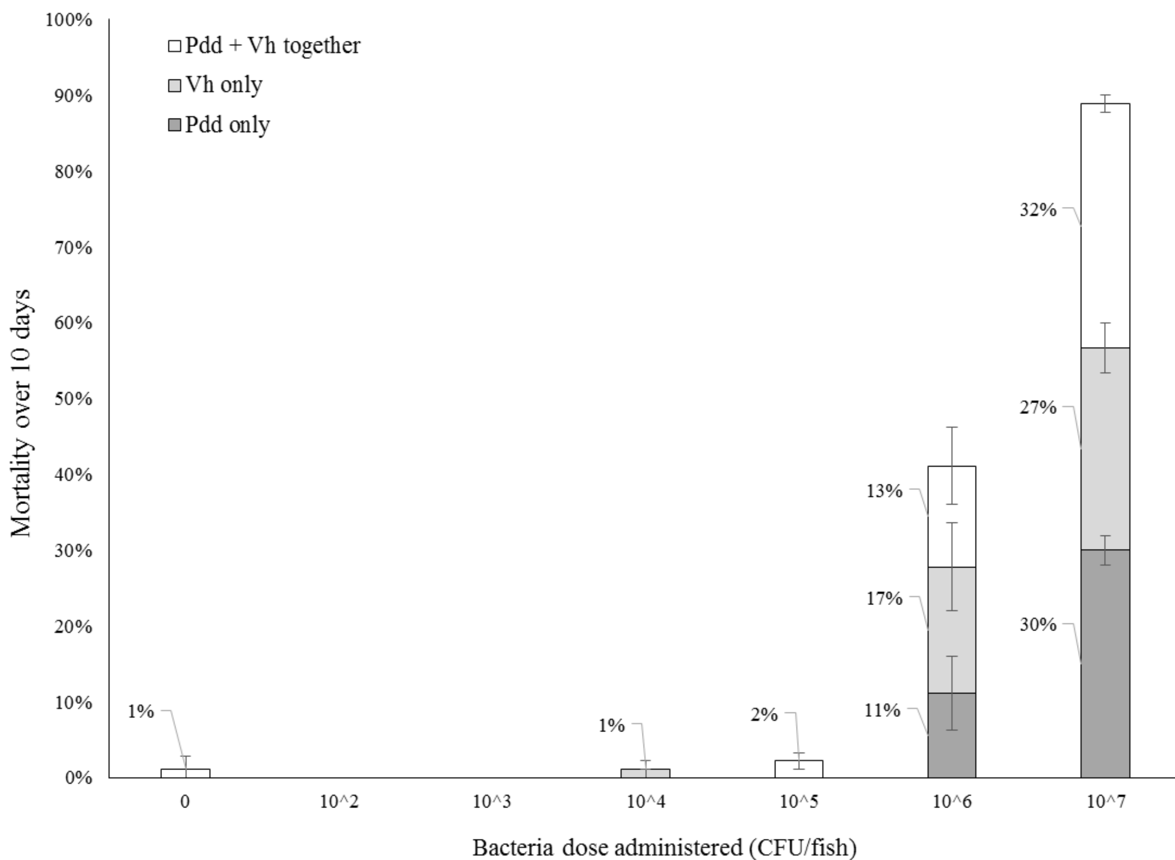


Figure 32: The effect of challenge dose and bacterial strain on mortality of YTK.

During the second trial, fish challenged with the plasmid-bearing Pdd strain (AS-16-0963#3) experienced 47% mortality over 10 days when challenged with 10^4 CFU/fish. This compares with 0% mortality when the fish were challenged with the same dose of low-virulence Pdd in trial 1. When this same high virulence Pdd (AS-16-0963#3) was co-administered with *Vibrio harveyi* (AS-17-6320#3), no mortality occurred over 10 days. The reason for this unexpected result is not clear and requires further investigation, however it does agree with our previous trial in that the two bacteria do not interact to increase virulence.

3.i. Gene expression

Expression of the *Vibrio harveyi* hemolysin gene (Vhh) when *Vibrio harveyi* isolate AS-18-2495#1 was grown in combination with each of the five plasmid bearing Pdd isolates was analysed with RT-qPCR. Results showed significant up-regulation of the Vhh gene when the *Vibrio harveyi* isolate was co-cultured with only with Pdd strain number AS-16-2454#19 (Figure 33). Further *in vivo* challenge work is required to determine whether this increase in activity of the Vhh gene relates to increased mortality when fish are challenged with both bacteria.

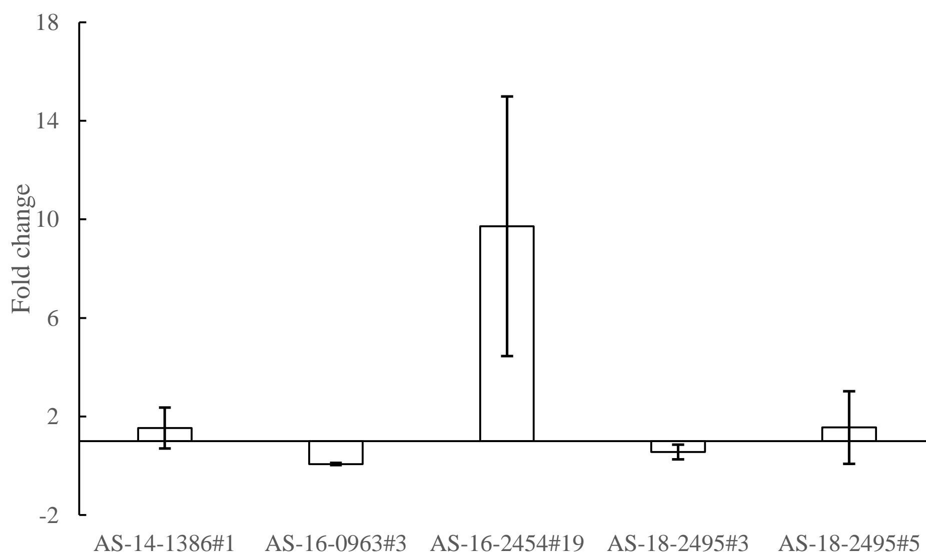


Figure 33: Relative transcription of hemolysin gene, *Vhh* when *Vibrio harveyi* isolate AS-18-2495#1 was grown in combination with plasmid bearing pdd isolates

Implications

Implications from each objective are listed below.

1. *Optimise the use of hydrogen peroxide to treat flukes in warm water.*

    - Industry have adjusted their protocols for the use of hydrogen peroxide based on the outcomes of this project to provide a greater margin of safety in the use of hydrogen peroxide in warm water.

2. *Investigate alternative fluke management methods to hydrogen peroxide in warm water.*

    - Based on the results of the trials reported here, industry and researchers are investigating further the use of herbal extracts for managing monogenean flukes. We have engaged another honours student to look at the commercial application of this approach under FRDC2017-030 whose work will include on-farm work.

    - Our results demonstrate that low salinity bathing may be a practical solution to fluke management once the industry grows to a size where well-boats are used.

    - In collaboration with UWA and industry, further efficacy trials are planned for the palatable PZQ formulation developed under this project. UWA and DPIRD have secured another UWA Pathfinder grant to investigate commercialisation options and we are investigating IP protection options for this formulation.

3. *Quantify the benefits of an autogenous vaccine against* Photobacterium damselae *subspecies* damselae.

    - Under this project we have proven that the autogenous vaccine currently in use by industry is effective at generating a strong antibody response against the two strains of bacteria contained within this vaccine.

4. *Characterisation of Australian strains of* Photobacterium damselae *subspecies* damselae *and* Vibrio harveyi.

    - Data generated on the characteristics of different strains of Pdd and Vh in this project are now being utilised under FRDC 2018-101 to ensure effective vaccines are formulated for industry against the most virulent strains of these bacteria.

5. *Determine in vitro whether interactions exist between* Photobacterium damselae *subspecies* damselae *and* Vibrio harveyi *that influence virulence.*

    - No evidence was found of interactions between Pdd and Vh that influence virulence.

Recommendations

As outlined throughout this report, activities are already well underway to further develop all aspects of those findings worthy of further investigation. Some findings are already being used directly for commercial benefit whilst others are being used to inform other current FRDC projects.

Extension and Adoption

Based on the project results, industry have already adopted modified bathing protocols.

Under FRDC2017-030 an honours student will be investigating the commercial use of herbal extracts to aid in parasite management.

The findings relating to the characterisation of *Photobacterium* and *Vibrio* have been extended to our research partners under FRDC 2018-101 for developing new and improved vaccines against these bacteria.

Project materials developed

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Gupta, et al. (In prep). Phenotypic and molecular characterization and pathogenesis of *Photobacterium damselae* subsp. *damselae* infections in Australian yellowtail kingfish (*Seriola lalandi*).

Appendices

Appendix 1 - Project staff

Dr Gavin Partridge – DPIRD Principal Research Scientist (Marine Finfish). Principal Investigator

Dr Lindsey Woolley – DPIRD Research Scientist (Marine Finfish). Co-investigator

Mr Luke Pilmer – DPIRD Research Technician (Marine Finfish) and PhD student

Dr Nicky Buller – DPIRD Senior Microbiologist. Co-investigator

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Ms Erica Starling – Indian Ocean Fresh Australia, Managing Director

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Mr Bryn Warnock – Indian Ocean Fresh Australia, Technical Supervisor

Associate Professor Alan Lymbery – Murdoch University (Parasitology)

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Ms Joan Gao – Curtin University, Masters student

Mr Jay Anderson – The University of Queensland, Honours student

Appendix 2 - Intellectual Property

UWA and DPIRD are currently investigating options for IP protection of the palatable PZQ formulation that was partly funded through this project.

Appendix 3 – References

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APPENDIX 4 – Woolley et al. Submitted

Effect of temperature and repeated hydrogen peroxide treatments on yellowtail kingfish *Seriola lalandi*

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Abstract

Hydrogen peroxide is routinely used in commercial yellowtail kingfish (YTK) culture to treat ectoparasitic infections. This study aimed to determine the acute effects of a repeated exposure to hydrogen peroxide on YTK at two rearing temperatures, 18 and 26 °C. The repeat bathe procedure occurred 14 days after the initial bathe, this is in line with commercial realities of ectoparasitic treatment regimes. Three concentrations of peroxide were trialled, 85 mg L^{-1}, 170 mg L^{-1} and 340 mg L^{-1}, with bathing conducted over 30 minutes. Survival was 100% in the majority of treatment tanks, with 30% mortality occurring in a 26 °C, 340 mg L^{-1} tank following their second bathe.

Blood biochemistry and haematology analyses revealed there were no acute effects of peroxide concentration on any of the blood parameters. Serum and mucous lysozyme were unaffected by temperature, peroxide dose and the interaction of these terms. Plasma protein thiol oxidation and mucous mapping were found to more sensitive tools for detecting stress in individuals treated at the highest peroxide concertation. There was a significant effect of both water temperature and hydrogen peroxide concentration on the percentage of oxidised plasma protein thiols. Fish bathed at 340 mg L^{-1} had a significantly higher percentage of protein thiol oxidation than those bathed at 85 mg L^{-1} and the control. The highest peroxide dose also significantly reduced the size and abundance of common mucous cells within the skin epithelium following the repeat bathe.

Therefore, despite evidence that 340 mg L^{-1} of hydrogen peroxide for 30 minutes is very close to the lethal limit for YTK in warm water, this was not evident in the majority of measured stress parameters, with the exception of protein thiol oxidation and skin mucocyte data. Whilst YTK appeared to be relatively tolerant of the high concentration of peroxide tested in the current study, these fish were not infected with flukes, and any burden of fluke infection is likely to impact the overall health of the fish making them more susceptible to the acute stresses of peroxide bathing.

Introduction

As production of the carangids genus *Seriola* through open sea cage culture rapidly expands in temperate areas such as Mexico, Chile, the United States, New Zealand, Japan and Australia (Purcell et al., 2016; Sicuro and Luzzana, 2016), effective health management strategies must be developed to limit the damaging effects of parasitic infections. Open cage culture systems allow harmful parasites to be freely transmitted among fish in the cages through the unrestricted exchange of water. The three major ectoparasitic monogeneans affecting yellowtail kingfish *Seriola lalandi* in Australia are the skin parasites, *Neobenedenia girellae* and *Benedenia seriolae*, and the gill parasite, *Zeuxapta seriolae* (Hutson et al., 2007). The fecundity and longevity of monogeneans has been well studied in farmed fish. The lifespan is largely affected by water temperature, with maturation times decreasing with increasing water temperatures. For example, in warmer waters, *Benedenia seriolae* begin producing eggs within 14 days of attachment at 26 °C compared to 25 to 41 days at temperatures below 17.5 °C (Lackenby et al., 2007). Mortality of farmed fish is the obvious risk and some infections have been known to depopulate entire cages, however sub-lethal effects such as stunted growth and effects on marketability due to the appearance cause severe economic loses to industry (Reyes-Bercerril et al., 2017). Parasite management, whilst critical, is costly, time consuming, labour intensive and potentially lethal if administered incorrectly.

Hydrogen peroxide has been used in aquaculture over the past three decades in the treatment of ectoparasites and was first used to remove sea lice infections from farmed Atlantic salmon (Treasurer and Grant, 1997). Hydrogen peroxide is widely used in the *Seriola* industry as it effectively controls both gill and skin fluke infections. The mode of action is largely unknown but it is thought to cause paralysis of the parasite, triggering the parasite adaptors to detach from the host fish during a bathing treatment. Peroxide bathing is highly effective at killing adult fluke but ineffective at preventing reinfection as mature adult parasites release resistant eggs during a bathe which causes reinfection of the cage (Ernst et al., 2005). Due to the reproduction of the fluke, cyclical treatment is needed to prevent re-infestation and amplification of the infection of the cage population. Therefore, second consecutive bathings are timed to kill newly hatched juveniles before they reach sexual maturity and release new eggs, thereby breaking the parasite life cycle (Ernst et al., 2005; Fensham et al., 2018).

Hydrogen peroxide is readily available, oxidises into non-toxic substances, and is convenient to use on harvestable-sized fish as it has no withholding period. However, the toxicity of

hydrogen peroxide to fish is known to increase with increases in treatment concentration, time of exposure and water temperature (Isshiki et al., 2007; Rach et al., 1997). Large mortality can occur when hydrogen peroxide is used at incorrect concentrations and/or inadequately mixed in the cage (Isshiki et al., 2007). Conventional bathing treatment concentrations are reported to be as high as 300 mg L^{-1} in Japan (Hirazawa et al., 2017) and 186 mg L^{-1} in Australia (Fensham et al., 2018). Bathing concentrations need to be high enough, and exposure times long enough, to effectively remove parasites without causing ill effects to the fish.

There is much literature on the susceptibility of parasites, and host sensitivity, to hydrogen peroxide however this is the first study investigating the stress response of *Seriola lalandi* to the commercial realities of repeated bathing in line with parasitic life cycles. The present study aimed to determine the acute effects of repeated exposure to hydrogen peroxide on yellowtail kingfish on physiological measurements of stress. Three hydrogen peroxide concentrations were tested, 85, 170 and 340 mg L^{-1}, with fish held at two water temperatures, 18 and 26 °C. Fish were bathed in hydrogen peroxide twice with 14 days between each event, in line with common industry practice. The stress response of the fish was measured through blood analysis, histological investigation of gills, skin and gut and immune function parameters after each bathing event.

Methods

Seriola lalandi (yellowtail kingfish) were held in 180 L tanks. All treatments were conducted in triplicate, except for the control treatments which were tested in duplicate due to tank availability. Each tank was stocked with 10 fish (116 ± 17g) at ambient water temperature (18 °C). Individual body weight was recorded, and each fish was implanted with an RFID tag in the left shoulder. Water in the tanks assigned to warm water treatments, was gradually increased to 26 °C over a three-day period. During both the acclimation and treatment periods, all fish were fed to satiety once per day on a commercial diet.

Two hydrogen peroxide bathing events were conducted. The initial bathe was conducted after the 3-day acclimation period (Day 0) and the second bathe, 14 days after the initial bathe (Day 14). During each bathing event, the water volume in each tank was decreased to 80 litres to allow for rapid dilution of the peroxide at the end of the 30 minute bathe period. Each

tank was rapidly flushed for 2 minutes with either ambient or warm water at a rate of 120 litres per minute. After rapid flushing, flow resumed at 3 litres per minute.

During the bathing event, tanks were static and diffused oxygen was added to each tank. Dissolved oxygen and temperature were measured at the beginning and at the end of the bathe. Hydrogen peroxide (Jasol™, 50%; w/w) was added gravimetrically to each tank to achieve the target concentration, with concentrations then determined potentiometrically by titrating against potassium permanganate. Two titrations were conducted on each tank, the first 5 minutes after the bathe commenced and the second at 25 minutes. The weights of hydrogen peroxide used in each treatment were 13.5 grams (85 mg L^{-1}), 27 grams (170 mg L^{-1}) and 54 grams (340 mg L^{-1}). In order to prevent 'hot spotting', the required weight of peroxide was diluted into 10 litres of the tank water and mixed prior to being added. Central aeration in each tank further mixed the peroxide during the bathe.

Bathing times were staggered to allow fish to be sampled immediately after the 30 minute immersion. After each bathing event, designated sampling fish were euthanised using 100 mg L^{-1} of MS-222 and sampled for blood and/or organs (Table 1). Whole blood was taken from the caudal vein of each sampled fish using lithium heparin (Heparin salts, Sigma-Aldrich) coated needles, stored in 1 mL foetal tubes (Minicollect tubes LH; Greiner Bio-One, Austria) and stored at 4 °C overnight prior to processing. Foetal tubes were spun for 3 minutes at 1000 *g*, 4 °C. Blood plasma was collected and divided into duplicate 50 µL aliquots for measurement of osmolality using a cryoscopic osmometer (Osmomat® 030), with the remainder being frozen separately prior to analysis for blood biochemistry using an AU 680 Clinical Chemistry analyser (Beckman Coulter Inc., Ireland), including analysis of Na, K, Cl, Mg, Ca and urea. Samples of the gill, midgut and skin were dissected from each of the two euthanised fish. The sections were fixed in 10% neutral buffered formalin for traditional histological examination. Histology sections were stained in H&E and PAS-Alcian Blue for mucous cell counts, and determination of villi height in the gut and of the thickness of the gut muscularis and skin.

On Day 7, an additional seven fish were added to each of the two control tanks. This allowed for additional sampling from the control treatments for haematology and mucous mapping following the repeat bathe on Day 14.

Following the second bathe, individual body weight was recorded for all fish and two fish per treatment tank were euthanised and sampled. The same sampling protocol was completed

following the second bathe, with the exception that three fish were sampled from each of the two control tanks which were bled. Additional skin samples were taken from the fish sampled in the 340 mg L^{-1} treatment at each temperature and their respective control tanks for mucosal assessment by Quantidoc® (Norway) following methods outlined in Pittman et al. (2013). A skin section, 3 × 2 cm, was sampled adjacent to the pectoral fin and below the lateral line. The sections were placed in histology cassettes and fixed in 10% neutral buffered formalin prior to processing. Skin sections were used for the determination of mucous cell count and cell size. The ratio of the cell size and density are linearly correlated in control fish, and so are combined to a single metric value which serves as a functional proxy for 'barrier strength' (Pittman et al., 2013).

Immune function measurements of plasma protein thiol oxidation, serum and skin mucous lysozyme were completed 48 hours post the second bathe (Day 16). Three replicate fish per tank were euthanised. Whole blood was collected from the caudal vein using lithium heparin (Heparin salts, Sigma-Aldrich) coated needles, and stored in 1 mL foetal tubes (Minicollect tubes LH; Greiner Bio-One, Austria). Immediately after collection, 90 µL of whole blood was taken for the measurement of plasma protein thiol oxidation and the remaining was utilised for running a standard panel of haematological parameters (i.e. complete blood count, CBC).

Analysis of plasma protein thiol oxidation was performed through a modified method as previously described by (Lim et al., 2020), the malpeg assay. Briefly, two aliquots of 45 µL whole blood were added to separate 1.5 ml microfuge tubes. One aliquot was treated as a 'control' and the second aliquot was 'trapped' for its oxidative state by adding a trapping solution containing 40 mM imidazole, 154 mM NaCl, 62.5 mM 5000 g/mol polyethylene glycol maleimide (JenKem Technology, USA). Tubes were briefly vortexed and centrifuged at 3000 *g* for 10 minutes with the supernatant containing the plasma collected. The malpeg assay was performed as described by Lim et al. (2020). In order to estimate the molecular weight of the unidentified protein band (Figure 1a), the relative migration distance of the proteins (R_f) calculations was used. The percentage of protein thiol oxidation was calculated using the densitometry of the control and trapped samples (Figure 1b) shown in Equation 1:

$$Protein\ thiol\ oxidation\ (\%) = \frac{Trapped\ Sample\ densitometry\ (a.u.)}{Control\ sample\ densitometry\ (a.u.)}\ X\ 100 \qquad \text{(Equation 1)}$$

The following haematology parameters were determined; haemoglobin (HB), haematocrit (HCT), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin content (MCHC), mean corpuscular haemoglobin (MCH) and white blood cells

(WBC). Red blood cell count was determined from heparinised whole blood using the CELL-DYN 37000 haematology analyser (Abbott Laboratories, Abbott Park, IL, USA) using the resistant red cell mode. This analyser has been validated for non-mammalian species, but no method comparison studies have been performed in *Seriola lalandi* (Vap et al. 2012). As *Seriola lalandi* have nucleated red blood cells, and cell lysis is needed for haemoglobin determination, red blood cells were lysed by diluting whole blood 1:1 with distilled water before centrifugation to remove nuclear debris. Haemoglobin content was performed on the CELL-DYN 3700 using a modified haemoglobin-hydroxylamine method (Latimer, 2011). Haemoglobin concentration was multiplied by the dilution factor to give total haemoglobin. PCV was measured by centrifugation at 10 000 *g* for 6 minutes (Hettich haematocrit centrifuge, Hettich, Tuttlingen, Germany). The indices MCV, MCH and MCHC were calculated from data determined according to the above methodology.

Leukocyte count was performed using a method described in avian species, which also have nucleated red blood cells (Lane 1991). Briefly, blood films were prepared and stained using an automated stainer (Haematek slide stainer, Ames Company, In, USA) with HaematekStain Pak Wright-Giemsa stain. An estimated leukocyte count was performed on each smear in the area of the monolayer where there appeared to be an even distribution of leukocytes. The average number of cells were determined by examining ten fields on 40 x magnification, this was multiplied by a factor of 2000, and converted to cell L^{-1}.

Further samples of whole blood, without anticoagulant, were collected and stored in 2 mL Eppendorf tubes. The blood was allowed to clot at room temperature for 1 hour then stored at 4 °C overnight prior to processing. The samples were spun at 1000 *g* for 10 minutes, serum removed and stored at -80 °C until analysed.

Lysozyme activity was measured in serum and skin mucus using a turbidimetric assay, measuring the lysis rate of *Micrococcus luteus* (Ellis, 1990). Briefly, to determine lysozyme activity in serum and mucus, respectively 175 and 150 µL of 0.075% *M. lysodeikticus* was prepared in 2 mM phosphate citric buffer (PCB, pH 6.2). Skin mucous was collected from each euthanised fish by scraping the entire surface of both sides of the body with the back of a #15 surgical blade. The mucous was transferred into tubes containing 1 mL PBS, pH 7.4. To remove the cellular debris from mucus, the homogenate was centrifuged at 25 000 *g*. The supernatant was stored at -80 °C for later determination of lysozyme. Protein content of the skin mucous

was determined using Bradford reagent (B6916, Sigma-Aldrich) following the manufacturer's protocol and the mucous lysozyme results expressed per mg of protein.

Results were analysed by two-way ANOVA to determine statistical differences between treatments for parameters of weight gain, CBC, sera and skin mucus lysozyme, plasma protein thiol oxidation, and mucous cell size and density. A full factorial ANOVA with *post-hoc* Tukey's test of multiple comparisons was used to determine the effect of repeated bathing, peroxide concentration and temperature treatments on the blood biochemistry and histological changes within the midgut, skin and gills. Data were normalised by arcsine transformed where needed. Significance was accepted at $P < 0.05$. The data were analysed using JMP software (Version 14, SAS Institute Inc.).

Results

Hydrogen peroxide dose

The measured concentrations of hydrogen peroxide in each treatment during each bathe are shown in Table 2. In all treatments, the actual concentration of hydrogen peroxide dose measured in each tank was higher than the target concentration dose. The overall average percentage by which the target dosage was overshot, was 6%.

Fish growth

At the start of the trial, the average fish weight across all tanks was 116 ± 2 grams. After 14 days, fish held in ambient water tanks had grown by 62% to 188 ± 2 grams whereas, those in warm water tanks grew by 87% to average 217 ± 4 grams. The effect of hydrogen peroxide dose and water temperature on weight gain were analysed via two-way ANOVA. It was not possible to include weight gain from control fish into the analysis, as those additional fish added to the tanks on Day 7 were not weighed. As expected, there was a significant effect of water temperature on growth, with fish at warmer temperatures (26 °C), having a greater weight gain than those at ambient temperatures (18 °C) ($P < 0.001$). Whist there was a trend of fish bathed at 85 mg L^{-1} being larger than those bathed at both 170 and 340 mg L^{-1} at both temperatures, there was no significant difference ($P = 0.40$).

Survival

Survival in majority of the treatment tanks was 100%. One fish died within 3 hours following the first bathe in the ambient, 340 mg L^{-1} treatment tank. The concentration of peroxide in this replicate tank which resulted in one mortality (355 mg L^{-1}), was similar to the other two tanks (352 and 354 mg L^{-1}) however, this tank did have the lowest average dissolved oxygen of the three tanks (9.9 vs. 12.3 and 12.6 mg L^{-1}). Another fish from an ambient 85 mg L^{-1} hydrogen peroxide treatment died 8 days after the first bath, therefore the death was unlikely as a result of the bathe itself. Immediately following the second bathe, three of the remaining six fish in one of the warm water, 340 mg L^{-1} replicate tanks died, and the three surviving fish displayed very slow swimming. Survival in the other two replicate tanks of the same treatment was 100% and all fish in these two tanks displayed normal swimming. The three dead fish were sampled immediately for blood biochemistry as well as histology. As a result of this high variation in survival between replicates, there was no effect of peroxide dose (P = 0.369) nor water temperature (P = 1.00) on survival following the second bathe however, the mortality is still of commercial relevance.

Blood Biochemistry

Blood biochemical analyses are shown in Table 3. This data set excludes those deceased fish sampled as described above and these data are discussed separately below. The data set was analysed by a full factorial ANOVA (Table 4). The results of this analysis demonstrated consistent significantly higher levels of urea, potassium, calcium and magnesium in fish held in warm water relative to those in ambient water. However, there were no significant effects of peroxide concentration (or the interaction of peroxide concentration and water temperature) on any of the blood biochemical parameters.

Comparison of the blood biochemical data of the three moribund fish collected from the 340 mg L^{-1} treatment in warm water against those surviving fish in the other two replicate tanks of the same treatment on Day 14, showed significant elevations in a number of parameters in the moribund blood compared to surviving fish blood; namely: sodium ($P < 0.01$) and magnesium ($P = 0.02$; Table 5). There was also an elevation in blood chloride levels (moribund, 191 mM; control, 153 mM) however, this was not statistically significant (P = 0.06). Blood urea ($P = 0.61$), potassium ($P = 0.44$) and calcium ($P = 0.82$) blood levels were not affected by the health status of the fish. The single fish sampled immediately at the end of

the bathe from the replicate tank in which the mortality occurred, had an extremely high level of potassium (31.2 mM), and it is likely that this fish would have died if it had not been sampled. However, this fish had normal healthy blood levels of sodium (181 mM), chloride (171 mM) and magnesium (2.53 mM).

Blood osmolality was not significantly affected by peroxide dosage at Day 0 or Day 14 (P = 0.25; Figure 2) however, fish in warm water tanks had significantly higher blood osmolality (463 ± 7.0 mmol kg^{-1}) compared to fish in ambient water tanks (444 ± 6.5 mmol kg^{-1}, P = 0.01; Figure 2). There was no significant difference in blood osmolality between surviving and moribund fish in the warm, 340 mg L^{-1} treatment tanks after the second bathe (P = 0.15; Table 5).

Haematology

Haematology data collected from fish two days after the second bathe are shown in Table 6. Two-way ANVOA revealed no effects of water temperature or hydrogen peroxide dose on the majority of parameters with the exception of haematocrit which was found to be affected by dose and the interaction of dose and temperature. White blood cell counts were found to be effected by temperature, with fish held in the warm water having significantly lower WBC count ($20 \pm 2 \times 10^9$ L^{-1}) compared to those held in ambient water ($28 \pm 2 \times 10^9$ L^{-1}; P = 0.02).

Histology

The number of mucous cells in the midgut was affected by temperature, with fish held in warm water having significantly more mucous cells (6.56 ± 0.12 mucous cells per 100 μm) compared to fish held in ambient water (5.95 ± 0.20 mucous cells per 100 μm; P = 0.01). There were no effects of peroxide dose or repeated bathing on the number of mucous cells within the midgut ($P > 0.05$). Both peroxide concentration (P = 0.009) and repeated bathing ($P < 0.001$) had a significant effect on midgut villi length, with the midgut villi length being significantly shorter in the fish treated with the 340 mg L^{-1} peroxide dosage compared to those treated at the 85 and 170 mg L^{-1} doses. The midgut muscularis thickness was significantly affected by repeated exposure to peroxide ($P < 0.001$), with the thickness increasing after the second bathe across all experimental tanks.

Similarly, as in the midgut, the number of mucus cells per gill filament were significantly impacted by water temperature, with fish held in ambient water (0.81 ± 0.10 mucous cells per 100 μm) having significantly more mucous cells compared to those fish in the warm water (0.53 ± 0.09 mucous cells per 100 μm; $P = 0.002$). There was no significant effect of peroxide concentration on gill tissue in terms of the number of mucous cells. There was a trend of decreasing gill mucous cell numbers with an increase in peroxide concentration in fish held at both temperatures after the initial bathe. However, there was no evidence of repeated bathing being detrimental to gill mucous cell numbers by Day 14 ($P = 0.123$). Whilst there was no significant difference in the number of gill mucous cells between moribund and surviving fish, in the 340 mg L^{-1}, warm water tank after the second bathe, there was severe congestion of the lamellae and lifting of the gill epithelium in the moribund fish, with one moribund fish suffering from extensive telangiectasia.

Skin thickness was significantly impacted by the repeated peroxide bathing, but not by peroxide dose or water temperature. Skin thickness of fish held in all peroxide treatment tanks significantly increased between Day 0 and 14, but not in those held in control tanks ($P < 0.001$). The number of mucous cells in the skin also significantly increased between Day 0 and 14 ($P < 0.001$). There was a significant effect of both repeated bathing and water temperature, with fish held in the ambient temperature have significantly more mucous cells in the skin ($P < 0.001$). The fish treated at the highest peroxide concentration had significantly more mucous cells at Day 14 compared to Day 0 ($P = 0.012$). There was no significant difference in either the skin thickness nor the number of skin mucous cells between the moribund and surviving fish treated at 340 g L^{-1} after the second bathe ($P = 0.731$ and $P = 0.166$, respectively).

Skin Mucous Mapping

Two cell types were identified in the dorsal skin through mucous mapping performed on Day 14, the common mucous cell (47 – 103 μm^2) and a larger cell of unknown type (mucous associated skin cells; MAS, 151 – 308 μm^2). These MAS cells did not stain with PAS-AB and appeared generally below the surface layer and therefore were not identified as common mucous cells. The size of the common mucous cell was not significantly affected by temperature ($P = 0.51$) however, peroxide concentration significantly reduced the size of these cells in fish treated at 340mg L^{-1} compared to those in the control ($P = 0.01$). Similarly,

the density of the common mucous cell (% of mucus to epithelium) was significantly affected by peroxide concentration, with the control fish having significantly more cells compared to those treated at 340 mg L^{-1} (P = 0.02; Figure 3). The MAS cell size and density were not affected by either water temperature or peroxide treatment. There were no significant effects of either water temperature or peroxide concentration on barrier strength of common mucous and MAS cells in surviving fish. However, the moribund fish in the 340mg L^{-1}, warm water treatment had significantly lower mucous cell density (5.0 ± 1.7%) compared to the surviving fish in the same treatment (17.7 ± 0.2%, P = 0.01). Consequently, the barrier strength was significantly lower in the moribund fish compared to control (0 mg L^{-1}) fish (P = 0.01).

Oxidative Stress

Results found a fish plasma protein sensitive to thiol oxidation (Protein "X") with an estimated molecular weight of ~53 kDa (Figure 1a). Protein "X" showed a significant increase in the percentage of oxidised protein thiols with an increase in peroxide concentration (P = 0.006; Figure 4). Fish bathed in 340 mg L^{-1} peroxide, had the highest levels of protein thiol oxidation (Figure 4). Fish from control tanks, not exposed to peroxide, had significantly lower levels of protein thiol oxidation in the warm water tank fish compared to ambient tank fish (P = 0.01; Figure 4).

Lysozyme Activity

The serum and mucosal lysozyme activity measured 48 hours after the second bathe are shown in Figure 5. A two-way ANOVA suggested no effect of peroxide dose (P = 0.55) or temperature (P = 0.14) on serum lysozyme. There was no significant effect of either peroxide dose (P = 0.80) or water temperature (P = 0.42) on the lysozyme activity within the skin mucous. However, there was a trend of increasing lysosome activity with an increase in peroxide dose.

Discussion

Hydrogen peroxide readily oxidises to safe by-products, does not require prescription from a veterinarian, and can be safely used on harvestable size fish without concern for withholding

periods prior to harvest, making it a very attractive treatment for parasitic control in farms. However, the consequences of an incorrect bathe can be dire with industry suffering significant stock losses from incorrect peroxide concentrations and or exposure time during bathing. High mortality (> 75%) was reported in *Seriola dumerili* and *Seriola quinqueradiata* treated at 300 mg L^{-1} for 60 minutes at 25 °C (Hirazawa et al., 2017). In the current study, survival was 100% in the majority of treatment tanks, with the exception of 30% mortality occurring in a single replicate treated at 340 mg L^{-1} at 26 °C following the second bathe. The reasons for the differences in survival between the replicates of this treatment are not certain, but they do suggest that this concentration of peroxide is close to the upper tolerable threshold and that repeat bathing has a greater impact on the fish than single bathing at this high concentration. Hirazawa et al. (2017) observed abnormal swimming behaviour and significant histopathological changes in the skin, gills and liver of fish treated at high concentrations of hydrogen peroxide. Abnormal (slow) swimming behaviour was noted in the fish treated at 340 mg L^{-1} immediately following the second bathe of this study. Hydrogen peroxide is an oxidising agent and gill tissue is particularly sensitive. The fact there was evidence of epithelial lifting of the gill lamellae in both the moribund and surviving fish treated at 340 mg L^{-1} in warm water again suggests that bathing at higher peroxide concentrations in the summer months requires particular close management in commercial applications. A previous study found that red spotted grouper bathed at 700 mg L^{-1} of peroxide resulted in mortality in fish that were bathed for 30 minutes at 25 °C, but not in fish bathed for 60 minutes at 10, 15 or 20 °C (Isshiki et al., 2007). In a similar study, Hirazawa et al. (2016a) treated healthy *Seriola dumerili* with an average weight of 208 g, at three peroxide concentrations of 75, 150 and 300 mg L^{-1} for 60 minutes at 25 °C. All but one fish died in the 300 mg L^{-1} treatment, whilst there we no other mortalities recorded for lower peroxide treatments in that study (Hirazawa et al. 2016a). Toxicity of peroxide against host fish was noticeable at 25 °C in both the Isshiki et al. (2007) and Hirazawa et al. (2016a) studies, as were the findings in this study and demonstrates higher peroxide toxicity with increasing concentrations in warm water.

Whilst the high peroxide concentration may cause some gill damage, the blood biochemistry parameters and lysozyme activity suggest the fish are able to mount a response to the stress of the high peroxide concentration. Blood biochemistry analyses revealed there were no acute effects of peroxide concentration on any of the blood biochemical parameters. Roque et al. (2010) found significant alterations in plasma ions, such as Na, Mg and Ca in sea bass exposed to hydrogen peroxide at 50 mg L^{-1}. The fish sampled from the 340 mg L^{-1} peroxide

treatment immediately following the second bathe, had extremely high levels of potassium. The average levels of Na (181 mM), Cl (171 mM) and Mg (2.53 mM) in surviving treatment and control (0 mg L^{-1}) fish, were not as high as those found in moribund fish. Thus, this suggests that the potassium imbalance found may be the primary cause of osmoregulatory failure, with disturbances in the other parameters following as a result of the potassium disruption and hence an increased chance of mortality in fish with high potassium levels. Haematological analysis revealed no effect of temperature or hydrogen peroxide dose on the majority of haematology parameters with the exception of haematocrit which was found to be effected by peroxide dose. Haematocrit levels increased significantly in fish treated with 85 and 170 mg L^{-1} of peroxide, in both ambient and warm water. Roque et al. (2010) found significant elevation in haematocrit level in sea bass treated with hydrogen peroxide at 50 mg L^{-1}. An elevation in haematocrit levels are the result of an increased oxygen supply (demand) to organs with an increase in metabolic activity or, an immediate stress response causing adrenergic splenic contraction. In this study, the haematological analyses were conducted 48 hours post the repeat bathe and any acute effects of bathing stress may well have resolved with Roque et al. (2010) finding most haematological parameters returned to normal levels after 24 hour following a peroxide bathe in sea bass treated at 50 mg L^{-1}.

Blood osmolality in teleost fish generally ranges from 280 to 360 mmol kg^{-1}, and is tightly regulated in a species-dependent range of salinities (Varsamos et al. 2005). Salmon treated with 500 mg L^{-1} hydrogen peroxide at 12 and 18 °C showed significantly increased plasma osmolality values compared to controls (Nowak et al. 2010). The elevated blood plasma osmolality indicates osmoregulatory stress, possibly due to gill damage resulting from exposure to the hydrogen peroxide. Elevated levels in salmon are described as greater than 378 mmol kg^{-1}, with 'normal' osmolality ranges between around 335 to 343 mmol kg^{-1} (Nowak et al. 2010). This study found a significant 4.3% increase in osmolality in warm compared to ambient temperature held fish. Whilst there was no significant effect of peroxide concentration, there was a trend of increasing blood osmolality with increasing peroxide bathing concentration. Fish treated in warm water at 340 mg L^{-1} had the highest levels of blood osmolality, with no significant difference found between surviving and moribund fish in this treatment. Osmolality levels were far higher in the current study than values reported by Mansell et al. (2005) for yellowtail kingfish treated with hydrogen peroxide at 300 mg L^{-1} for 10 minutes. In Mansell et al. (2005), blood osmolality significantly increased by 5.7%, from 380 mmol kg^{-1} pre-treatment to 400 mmol kg^{-1} post treatment, in yellowtail kingfish

held in variable ambient water (14 to 20 °C). In this study, the peroxide treatment duration was longer which may have caused an increase in blood osmolality, however the osmolality of the control fish are also considerably higher (420 – 440 mmol kg^{-1}). Similarly, Partridge et al., (2020) reported osmolality ranges of 420 to 460 mmol kg^{-1} in healthy *Seriola lalandi*. The cause of the increase in osmolality is largely unknown, however hypoventilation is known to occur during exposure to hydrogen peroxide, and any dysfunction of the ion regulation or respiratory epithelium may cause changes in the osmolality. However, there was no evidence of disruption to osmoregulation in the blood biochemistry in surviving fish. Hypoventilation is also known to occur when fish are held in oxygen supersaturated water, in both the current study and Partridge at al. (2020) study, the fish were held in systems supplied with oxygen saturated water which may explain the high levels of osmolality across the treatment tanks.

The variation in serum lysozyme activity between replicates in the control and 85 mg L^{-1} treated fish in warm water was high, demonstrating a variable lysozyme response in these fish. The lysozyme content of sera in fish held in warm water and bathed at 170 and 340 mg L^{-1} peroxide, was tight and low suggesting these fish were able to mount a strong lysozyme response to the stress at higher concentrations. These results are in contrast with what Hwang et al. (2016) observed on serum and mucus lysozyme activity of olive flounder exposed to hydrogen peroxide concentrations of 100, 300 and 500 mg L^{-1}. The serum lysozyme activity significantly increased across all peroxide treatments in the Hwang et al. (2016) study, however the levels in olive flounder lysozyme activity was 10-fold lower than those recorded in *Seriola lalandi*.

The mucosal associated lymphoid tissues in fish include the gut, skin and gills which have a mucus layer that forms the initial barrier to invasive pathogens. Repeated bathing of fish may strip these tissues of this mucus layer leaving them susceptible to invading pathogens and disease (Press and Evenson, 1999). Whilst there was no evidence of mucous cell stripping by repeated bathing in either the gill or skin tissue of surviving yellowtail kingfish in the present study, i.e. no significant differences in either the skin thickness or number of mucous cells in the skin between moribund and surviving fish, there were significant differences in the common mucous cell size and mucus cell density and hence 'barrier strength'. Barrier strength, being a proxy value, for the effects on cell size and density, may be a far more sensitive tool to determine the histopathological stressors on fish. The epithelium density of moribund fish was almost 30% lower than the surviving fish in the same treatment. *Seriola lalandi* infected with *Neobenedenia melleni* have a marked change to the skin tissue, with the

skin mucous cells almost undetectable and areas of the skin with absence of the mucous layer seen in infected fish (Reyes-Bercerril et al., 2017; Hirazawa et al. 2016b). This parasitic damage to the epidermis may facilitate further damage during a peroxide treatment. Further complication of effects of the fluke burden may cause fish to be further exposed to the oxidising effects of the peroxide leaving fish vulnerable to secondary infection by bacteria and viruses.

The current study utilised juvenile fish, 116 g, and in general, host toxicity of hydrogen peroxide depends on the life stage of the fish (Gaikowski et al., 1999). Shorter exposure times in combination with lower treatment concentrations have proven both safe and efficacious in *Seriola* culture (Hirazawa et al., 2016a). Hydrogen peroxide substantially reduced or eliminated infestations with *Neobenedenia* and *Zeuxapta* spp. on *S. dumerili*. Hirazawa et al., (2016a) found 75 mg L^{-1} hydrogen peroxide for 30 minutes completely eliminated *B. seriola*, *N. girellae* and *Z. japonica* infections in commercially cultured *S. dumerili* at a water temperature of 28.5 °C. Fensham et al. (2018) reported commercial treatment of *S. lalandi* for *B. seriolae* and *Z. seriolae* was 186 mg L^{-1} for 24 minutes in cool water (15.7 °C). This study supports the findings of Fensham et al. (2018) in that there are no acute health effects on *S. lalandi* repeatedly bathe with hydrogen peroxide at 170 mg L^{-1} for 30 minutes and further suggests that this regime is also safe in warmer water treatments.

Oxidative stress was assessed through a modified malpeg assay (Lim et al., 2020). This assay detects subtle changes in the reversible oxidative state of protein thiols which allows for the sensitive measurement of oxidative stress in tissue (Armstrong et al., 2011). This is the first time this method has been used in fish. This method found that fish exposed to hydrogen peroxide had a graded response in plasma protein thiol oxidation based on the dosage of peroxide. With increasing peroxide bathing doses, there was a significant increase in the level of plasma protein thiol oxidation. These results are supported by previous studies where exposure of fish to hydrogen peroxide altered the activity of antioxidant enzymes, increased plasma lipid peroxidation and increased plasma thiol oxidation (glutathione oxidation) in *Oncorhynchus mykiss* (Seker et al., 2015) and *Oreochromis niloticus* (Jia et al., 2019). An explanation of mechanism could be through the direct effect of peroxide being readily metabolised to hydroxyl radicals by Fenton reaction (Davies, 1995), which results in protein thiol oxidation in blood (Davies, 2016). Increased protein thiol oxidation can impact protein function and signalling, which can have impacts under both physiological and diseased conditions (Armstrong et al., 2011). The increase in protein thiol oxidation at high levels of

peroxide exposure, but not in other parameters measured in this study, suggests that Protein "X" thiol oxidation could be a novel fish blood biomarker for detecting exposure to chemical stress such as chemical treatments or pollutants.

The suggested water temperature for optimal growth in juvenile yellowtail kingfish is 26.5 °C (Abbink et al., 2012), and in the case of the present study, the warm condition was ~26.2 °C. This could explain in part, the significant reduction of protein thiol oxidation at warm water (~26.2 °C) as compared to ambient water (~18.7 °C) when fish were not exposed to peroxide. More studies are needed to further investigate the effect of temperature on oxidative stress.

The ability of the malpeg assay to track changes in protein thiol oxidation will facilitate studies examining the effect of infection or disease in fish. Interestingly, as classic measures in blood biochemistry and haematology analyses found little to no impact on health of the fish in the current study, this suggests that analysis of plasma protein thiol oxidation may be a more sensitive tool for measuring stress in individuals. Given that Protein "X" is a potential biomarker for fish health, further investigations into its identification are required.

In conclusion, despite evidence that 340 mg L^{-1} of hydrogen peroxide for 30 minutes is very close to the lethal threshold, this treatment regime did not result in significant changes in the majority of blood biochemistry or histopathological measurements in the fish which survived this treatment. There does appear to be an acute effect of high peroxide concentrations on the more sensitive physiological parameters of plasma protein thiol oxidation and mucocyte pathology. It is worth considering that any burden of fluke loading may impact the overall health of the fish and peroxide bathing in turn may compromise the fish. Concentrations of more than 340 mg L^{-1} at warm water temperatures would likely increase the risk of fish mortalities due to treatment toxicity.

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Table 1: Sampling conducted for the duration of the trial.

Day 0 Initial peroxide bathe	Day 1	Day 14 Repeated peroxide bathe	Day 16
Individual body weight [a]			Individual body weight [a]
Blood biochemistry	Blood biochemistry	Blood biochemistry	Blood biochemistry
Osmolality		Osmolality	Haematology
Histology		Histology	Serum lysozyme
		Skin Mucous Mapping [b]	Skin mucous lysozyme
			Plasma protein thiol oxidation
n = 2 fish tank^{-1}	n = 2 fish tank^{-1}	n = 2 fish tank^{-1} and 3 fish per control tank were bled.	n = 3 fish tank^{-1}

[a] Individual body weight recorded for all fish in each tank.

[b] Skin samples collected from fish held in the 340 mg L^{-1} peroxide treatment tanks (n = 2 fish tank^{-1}) at each temperature and their respective control tanks (n = 6 fish tank^{-1})

Table 2: Targeted and measured hydrogen peroxide concentrations for each bathing event.

Temperature	Target [H_2O_2] (mg L^{-1})	Day 0 Actual [H_2O_2] (mg L^{-1})	Day 0 % Difference	Day 14 Actual [H_2O_2] (mg L^{-1})	Day 14 % Difference
18.7 °C (Ambient)	0	-	-	-	-
	85	92.0 ± 1.3	8%	92.5 ± 0.5	9%
	170	179.8 ± 4.1	6%	178.7 ± 2.3	5%
	340	353.5 ± 5.7	4%	347.8 ± 1.2	2%
26.2 °C (Warm)	0	-	-	-	-
	85	96.6 ± 4.6	14%	90.6 ± 2.8	7%
	170	180.5 ± 3.8	6%	178.8 ± 7.9	5%
	340	352.0 ± 5.7	4%	341.3 ± 2.4	0%

Table 3: Biochemical analysis of plasma collected from yellowtail kingfish following hydrogen peroxide bathing on Day 0 and 14 at ambient (18 °C) and warm (26 °C) water temperatures. Values are mean mmol L^{-1} ± S.E.

			Urea	Na	K	Cl	Ca	Mg
Day 0	Ambient	0	4.5 ± 0.7	184.3 ± 3.0	1.6 ± 0.4	149.0 ± 4.0	3.2 ± 0.1	1.1 ± 0.1
		85	3.8 ± 0.2	183.0 ± 0.9	1.9 ± 0.1	143.0 ± 0.3	3.0 ± 0.1	1.2 ± 0.1
		170	5.2 ± 0.4	183.3 ± 8.4	1.7 ± 0.2	148.7 ± 8.6	3.0 ± 0.1	1.2 ± 0.1
		340	4.3 ± 0.4	190.7 ± 2.3	1.7 ± 0.1	156.0 ± 1.7	3.1 ± 0.1	1.3 ± 0.0
	Warm	0	8.8 ± 0.1	181.3 ± 5.0	3.9 ± 1.4	151.7 ± 4.0	3.2 ± 0.0	1.2 ± 0.2
		85	8.3 ± 0.7	188.0 ± 1.5	4.0 ± 0.2	153.0 ± 1.5	3.5 ± 0.2	1.4 ± 0.1
		170	8.7 ± 1.0	188.7 ± 2.8	1.8 ± 0.3	155.3 ± 1.5	3.5 ± 0.1	1.4 ± 0.0
		340	9.0 ± 0.8	190.7 ± 2.7	2.3 ± 1.3	157.0 ± 2.3	3.4 ± 0.1	1.4 ± 0.1
Day 1	Ambient	0	5.3 ± 0.2	187.3 ± 3.2	2.9 ± 0.6	155.0 ± 2.9	3.3 ± 0.1	1.1 ± 0.1
		85	4.9 ± 0.7	192.7 ± 2.0	2.6 ± 0.9	160.3 ± 1.2	3.3 ± 0.1	1.0 ± 0.0
		170	5.1 ± 0.8	184.3 ± 0.7	3.7 ± 0.5	153.7 ± 0.6	3.1 ± 0.1	1.0 ± 0.1
		340	4.5 ± 0.2	182.7 ± 1.2	3.2 ± 0.4	152.0 ± 1.0	2.9 ± 0.0	1.0 ± 0.0
	Warm	0	9.7 ± 1	187 ± 1.5	5.7 ± 0.1	160.7 ± 0.9	3.2 ± 0.1	1.1 ± 0.1
		85	6.5 ± 1.3	183.3 ± 3.3	4.5 ± 1	154.7 ± 2.8	3.2 ± 0.1	1.2 ± 0.1
		170	8.2 ± 1.7	182.7 ± 2	6.4 ± 0.3	158.3 ± 2.1	3.1 ± 0.1	1.2 ± 0.1
		340	7.8 ± 0.2	182.7 ± 6.1	5.8 ± 0.3	157.0 ± 5.6	3.2 ± 0.1	1.1 ± 0.1
Day 14	Ambient	0	4.7 ± 0.5	189.7 ± 2.0	1.5 ± 0.3	151.0 ± 0.5	3.4 ± 0.0	1.0 ± 0.0
		85	4.4 ± 0.1	188.0 ± 1.8	1.6 ± 0.0	147.5 ± 1.2	3.2 ± 0.0	1.2 ± 0.1
		170	5.8 ± 0.7	185.3 ± 1.7	1.5 ± 0.1	145.3 ± 1.2	3.2 ± 0.1	1.4 ± 0.0
		340	5.5 ± 0.3	184.7 ± 0.9	1.3 ± 0.2	146.0 ± 1.0	3.3 ± 0.0	1.4 ± 0.1
	Warm	0	8.4 ± 0.0	183.0 ± 0.0	7.2 ± 2.7	151.0 ± 1.0	3.3 ± 0.1	1.1 ± 0.1
		85	7.5 ± 0.4	184.0 ± 4.4	8.3 ± 0.6	149.0 ± 2.7	3.6 ± 0.2	1.4 ± 0.1
		170	8.0 ± 0.8	186.0 ± 3.5	2.9 ± 1.2	146.3 ± 2.9	3.6 ± 0.1	1.3 ± 0.0
		340	8.4 ± 1.1	185.3 ± 4	6.0 ± 2.3	152.7 ± 2.0	3.3 ± 0.2	1.3 ± 0.1
Day 16	Ambient	0	6.1 ± 0.5	185.7 ± 1.5	3.8 ± 0.3	152.7 ± 2.0	3.1 ± 0.0	1.7 ± 0.1
		85	5.5 ± 0.1	182.3 ± 8.1	2.6 ± 0.4	148.3 ± 11.7	3.1 ± 0.3	1.3 ± 0.0
		170	4.9 ± 0.3	194.3 ± 2.0	2.1 ± 0.2	161.3 ± 2.2	3.3 ± 0.1	1.1 ± 0.0
		340	5.0 ± 0.2	185.7 ± 2.9	1.7 ± 1.7	152.3 ± 0.9	3.1 ± 0.2	1.3 ± 0.3
	Warm	0	8.2 ± 0.8	187.0 ± 1.2	6.6 ± 0.8	161.0 ± 0.6	3.2 ± 0.1	1.9 ± 0.2
		85	6.8 ± 0.4	184.7 ± 3.5	5.9 ± 1.2	153.0 ± 1.3	3.4 ± 0.2	1.9 ± 0.2
		170	9.2 ± 0.4	193.0 ± 4.2	5.0 ± 2.3	162.3 ± 1.5	3.8 ± 0.1	2.2 ± 0.1
		340	7.8 ± 0.4	187.7 ± 7.0	6.1 ± 1.7	159.0 ± 3.0	3.5 ± 0.1	2.0 ± 0.2

Table 4: Analysis of variance (*P*-values) for the biochemical analyses of plasma data shown in Table 2.

		Urea	**Na**	**K**	**Cl**	**Ca**	**Mg**
Peroxide Dose	Day 0	0.64	0.28	0.22	0.23	0.77	0.23
	Day 1	0.27	0.23	0.14	0.58	0.22	0.81
	Day 14	0.31	0.98	0.29	0.08	0.89	0.99
	Day 16	0.23	0.11	0.57	0.13	0.15	0.84
Water Temperature	Day 0	**<0.0001**	0.58	**0.01**	0.09	**0.0002**	**0.04**
	Day 1	**0.0002**	0.19	**<0.0001**	0.21	0.80	**0.004**
	Day 14	**<0.0001**	0.21	**0.015**	0.10	**0.02**	0.27
	Day 16	**<0.0001**	0.71	**0.0012**	0.11	**0.005**	**0.002**
Dose x Temp	Day 0	0.80	0.68	0.24	0.70	0.06	0.78
	Day 1	0.52	0.37	0.89	0.14	0.13	0.77
	Day 14	0.73	0.36	0.29	0.69	0.10	0.26
	Day 16	**0.02**	0.96	0.89	0.85	0.63	0.14

Table 5: Electrolytes (mmol L^{-1}) and osmolality (mmol kg^{-1}) of plasma collected from control and moribund yellowtail kingfish following repeated hydrogen peroxide bathing at a peroxide dose of 340 mg L^{-1} held in warm water (26 °C).

Fish Status	Na	K	Cl	Ca	Mg	Osmolality
Moribund	210.3 ± 2.4	22.7 ± 8.1	193.0 ± 6.7	3.4 ± 0.1	4.7 ± 0.5	574 ± 40
Healthy	182.3 ± 8.2	15.2 ± 7.6	157.7 ± 6.1	3.4 ± 0.2	1.6 ± 0.7	502 ± 42
P	**< 0.01**	0.45	0.06	0.98	**0.02**	0.15

Table 6: Haematology parameters in yellowtail kingfish blood measured 48 hours after the second hydrogen peroxide bathe (Day 16).

	$[H_2O_2]$ mg L^{-1}	HB	HCT	PCV (%)	RBC	MCHC	MCH	MCV	WBC
Ambient	0	83 ± 3	0.54 ± 0.02	45 ± 3	3.4 ± 0.1	177 ± 1	24 ± 3	131 ± 1	42.3 ± 2.4
	85	72*	0.49 ± 0.04	44 ± 3	3.6 ± 0.2	144*	18*	120 ± 5	20.6 ± 5.8
	170	97*	0.51 ± 0.02	48 ± 4	3.8 ± 0.2	176*	23*	125 ± 4	27.8 ± 4.1
	340	74 ± 10	0.51 ± 0.02	43 ± 2	3.7 ± 0.1	169 ± 19	20 ± 3	117 ± 5	22.4 ± 3.6
Warm	0	73 ± 3	0.28 ± 0.02	46 ± 2	3.8 ± 0.1	158 ± 1	19 ± 1	121 ± 2	17 ± 4.2
	85	86 ± 16	0.58 ± 0.06	46 ± 5	3.8 ± 0.2	190 ± 41	23 ± 5	121 ± 5	20.6 ± 1.7
	170	93 ± 14	0.6 ± 0.04	47 ± 2	4 ± 0.1	198 ± 35	23 ± 4	117 ± 3	22.7 ± 6.1
	340	65 ± 7	0.42 ± 0.06	40 ± 6	2.8 ± 1	163 ± 8	29 ± 10	121 ± 1	18.3 ± 4
P_{dose}		0.5	**0.01**	0.81	0.61	0.77	0.94	0.52	0.2
$P_{temperature}$		0.93	0.14	0.69	0.28	0.63	0.55	0.43	**0.02**
P_{int}		0.87	**<0.01**	0.99	0.9	0.43	0.52	0.32	**0.04**

HB, haemoglobin per litre of whole blood (g L^{-1}); HCT, haematocrit (g g^{-1}); PCV, Packed cell volume (%); RBC, red blood cell (×10^{12} L^{-1}); MCHC, mean corpuscular haemoglobin concentration (g L^{-1}); MCH, mean corpuscular volume (pg); MCV, mean corpuscular volume (fL); WBC, white blood cells (×10^{9} L^{-1}). * n = 1 fish $tank^{-1}$ (insufficient sample for full analysis).

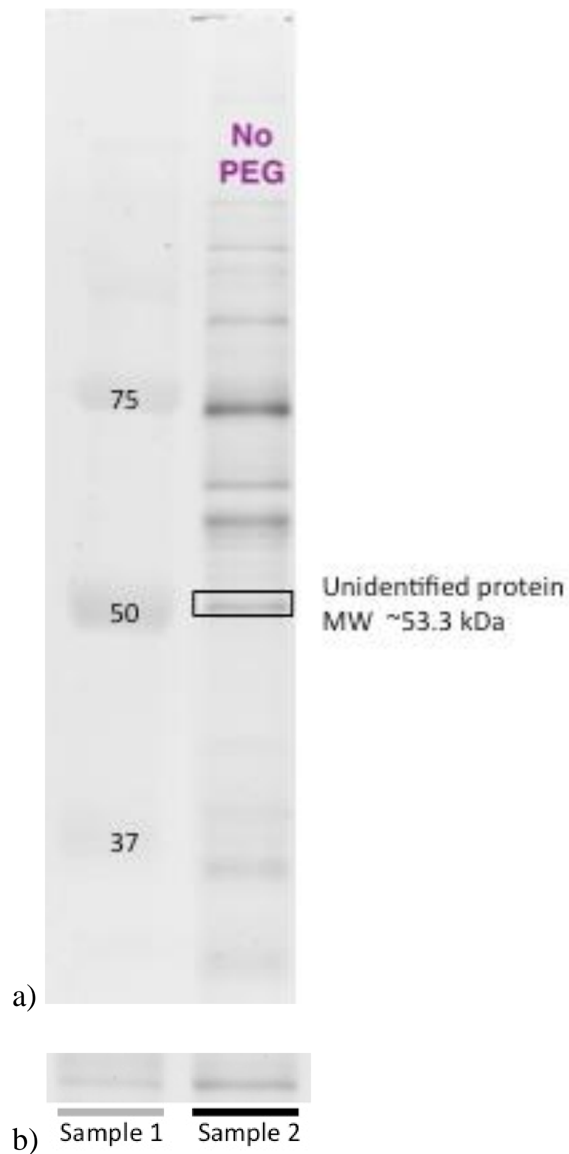


Figure 1. Illustration of Protein "X" position on an example gel image. a) Position of the unidentified protein band in the black box shows it at approximately 53.3kDa with the relevant molecular weight marker position adjacent to it, and b) sample 1 and 2 represent same sample protein bands, on the resolved gel image, with and without trapping solution respectively.

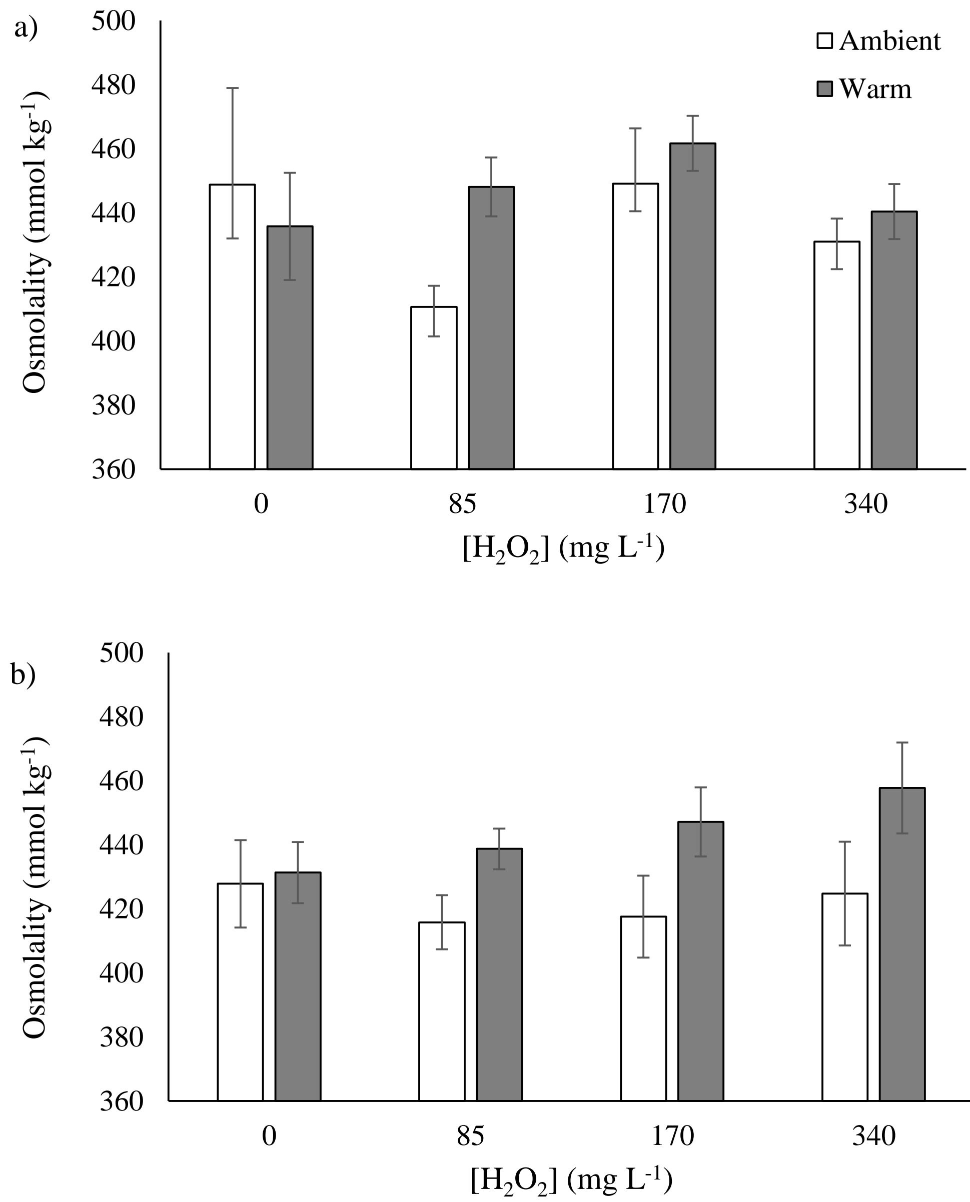


Figure 2. Blood osmolality (mean ± SE) of yellowtail kingfish immediately following the initial (a) Day 0, and the repeated hydrogen peroxide bathe (b) Day 14 at the various hydrogen peroxide bathing doses tested.

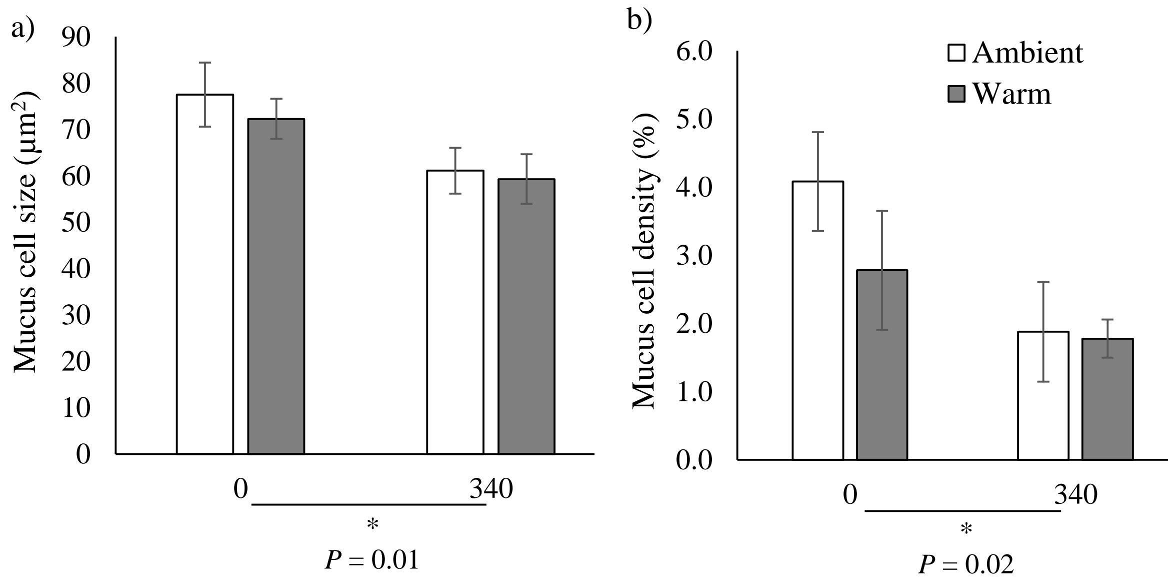


Figure 3. Effect of hydrogen peroxide dose (0 and 340 mg L^{-1}) and water temperature on a) the mucous cell size and b) density (as a % mucous to epithelium) within the skin epithelium of yellowtail kingfish subjected to repeated hydrogen peroxide bathing. Values are mean ± S.E.

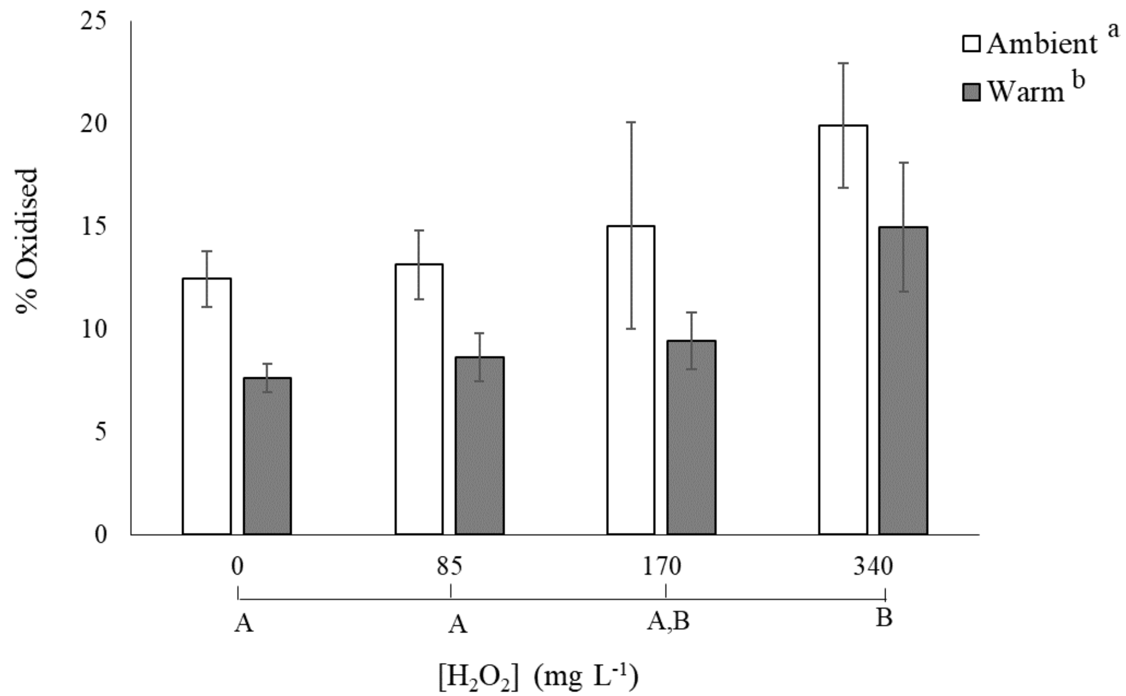
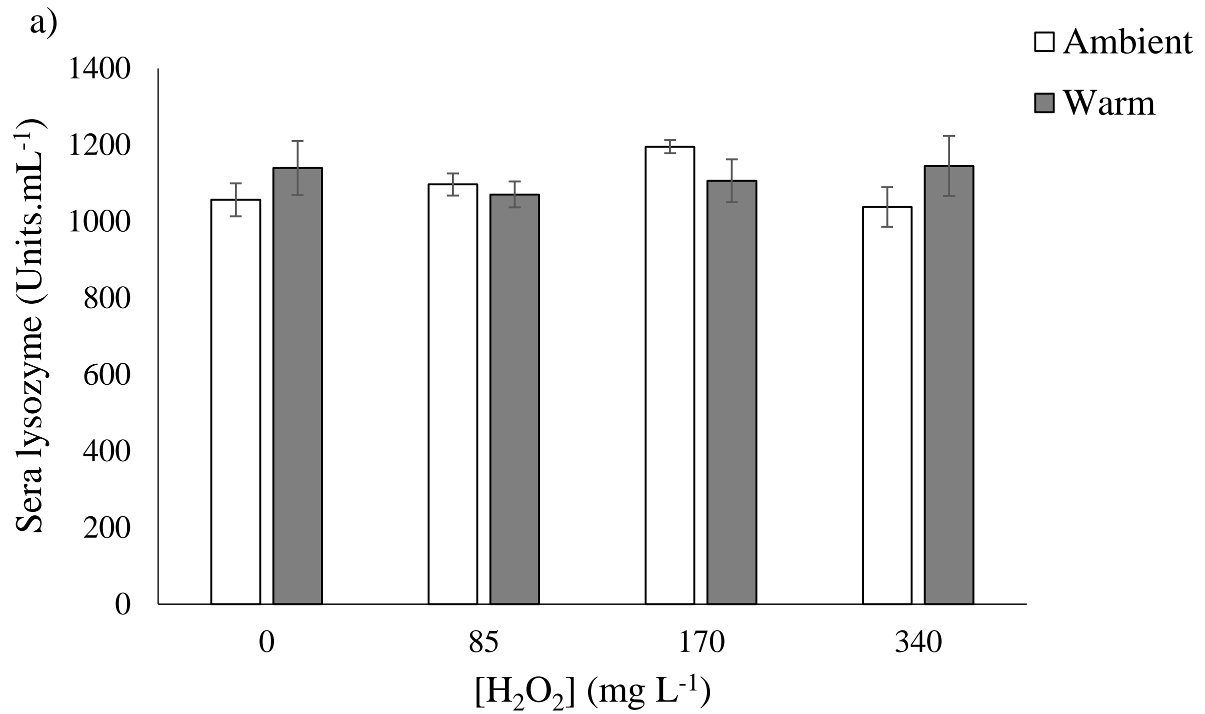


Figure 4. Oxidative stress measured as thiol oxidation of a plasma protein (% oxidised) 48 hours after the second peroxide bathe (Day 16). Different uppercase letters denote significant differences among the peroxide concentration treatments, and different lowercase letters denote significant differences among water temperatures. Values are mean ± S.E.



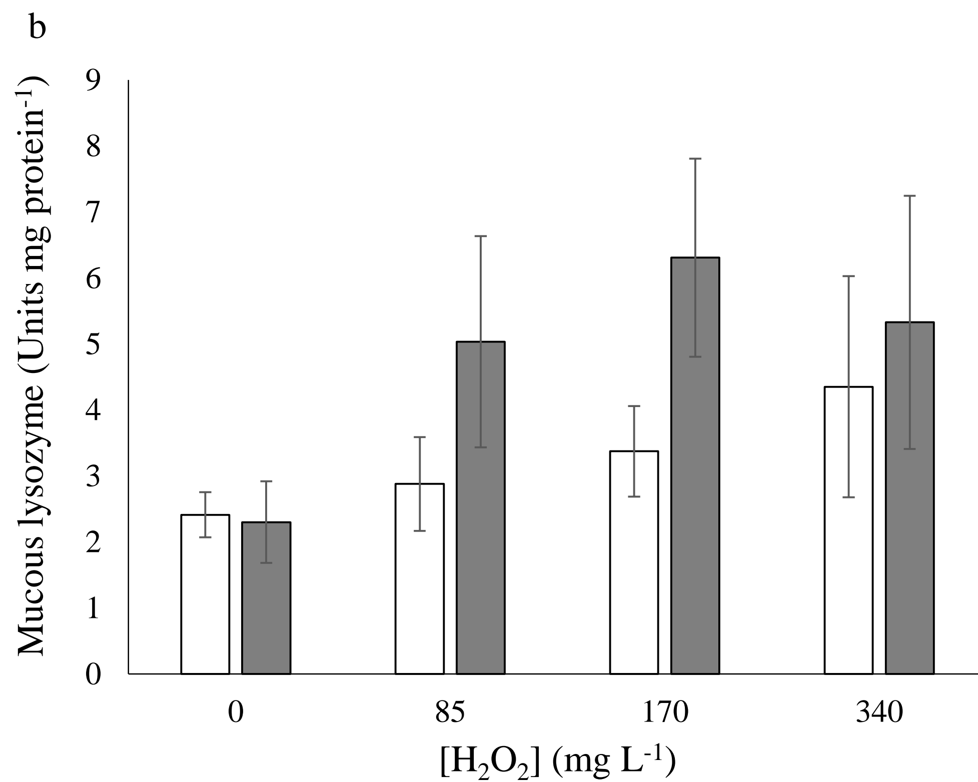


Figure 5. Lysozyme content in a) sera and b) mucous measured 48 hours after the repeated hydrogen peroxide bathe. Values are mean ± S.E.

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Appendix 5 - Joan Gao Master Thesis

School of Molecular and Life Sciences

The effects of garlic supplemented diets on *Neobenedenia girellae* attachment success on *Seriola lalandi*

Submitted by

10 Joan Gao

Submitted to

Jennifer McIlwain

Date: May 2018

1. Introduction

Pathogens and diseases are some of the biggest hurdles aquaculture faces today. Not only is the prevention and treatment of infections and diseases labour intensive, it is also costly and can be stressful to the fish (Bondad-Reantaso et al., 1995; Hutson et al., 2012). One pathogen that has been identified as a serious and chronic problem in the *Seriola lalandi* (yellowtail kingfish) culture industry is the monogenean *Neobenedenia girellae*, due to its host non-specificity, direct life cycle and the fact that it causes a wide range of health issues (Hirazawa et al., 2016a; Militz et al., 2013). Monogeneans are classified as flat worms that feed on mucous, blood and other bodily tissues, usually found on the external surfaces of a fish (Shirakashi and Hirano, 2015). Capsalid monogeneans such as *N. girellae* that require single hosts proliferate under aquaculture conditions where the hosts are stocked at high density and where infection transference is therefore aggravated (Morales-Serna et al., 2014). Severe *N. girellae* infections can cause skin lesions that leave the host susceptible to secondary microbial infections and other diseases and increased mortality rates (Hirazawa et al., 2016b).

Neobenedenia girellae has a direct life cycle with a single host. Free swimming oncomiracidia or juvenile *N. girellae* attach to the body of fish using haptors and graze on the hosts' epidermis (Hutson et al., 2012). As juveniles mature and develop into adults, eggs are laid into the water column on long filaments that get entangled on sea cages and other substrates (Hutson et al., 2012). The eggs are incubated in the environment and hatch into free-swimming, ciliated oncomiracidia for reinfection (Hutson et al., 2012). Due to the ease and effectiveness of reinfection, any treatment requires meticulous planning and is extremely labour intensive (Williams et al., 2007).

Currently avaliable treatments against monogeneans include bathing in hydrogen peroxide (H_2O_2) or freshwater and either bathing in or the oral administration of praziquantel. All of these treatments however offer only temporary relief as they are often non-lethal to eggs that are well protected by their sclerotised protein shells (Leef and Lee, 2009; Militz et al., 2014; Sharp et al., 2004; Shirakashi and Hirano, 2015; Williams et al., 2007). Thus, reinfection occurs almost immediately after treatment from untreated or unaffected eggs attached to the surrounding environment and sea cage nets (Ernst et al., 2005). Handling and treatment of the fish compounded with the stress of infection, do not come without risks and mortalities due to treatments or combinations of treatments can still result in mortalities (Williams et al., 2007).

Use of orally administered praziquantel has been used in treating monogeneans on Japanese yellowtail (*Seriola quinqueraduata*) and tiger puffer (*Takifugu rubripes*) and has been proven to be
50 effective (Hirazawa et al., 2000; Williams et al., 2007). Williams et al. (2007) and Partridge et al. (2014), trialled coating of feed pellets with praziquantel but found that palatability decreased effectiveness. Alternatives to orally administering antihelminthic treatments or chemical bathing is the use of dietary suppletmentation to treat and/or prevent infection. Examples include the use of seaweed extracts or the addition of garlic extract incorporated into feed pellets for immunostimulant effects (Hutson et al., 2012; Militz et al., 2013).

Garlic is known for a wide range of benefits to the immune system, including antibacterial, antifungal, antiviral, antiprotozoal and antiparasitic effects (Fridman et al., 2014; Harris et al., 2001). Additionally, garlic has been known to have beneficial effects on the immune system and cardiovasular system (Harris et al., 2001). The addition of dietary supplements such as garlic and
60 garlic extracts into commercial feed pellets could therefore be the first line of defence against monogeneans and other infections. Recent garlic studies on two fish species and two monogenean species have found positive results in both the treatment of monogeneans and reducing attachement rates (Fridman et al., 2014; Militz et al., 2014, 2013).

The aim of this study is to ascertain the effects of different garlic compounds on *Neobenednia girellae* attachement success on *Seriola lalandi* (yellowtail kingfish). A similar study was undertaken by Militz et al. (2013) on the effects garlic extract had on *N. girellae* infections on saltwater barramundi (*Lates calcarifer*). In this study they successfully reduced *N. girellae* attachment by 50% after feeding a diet supplemented with 50 mL/kg of garlic extract for 30 days. Thus, it is hypothesised that garlic supplemented feeds will significantly reduce *N. girellae* oncomiracidia
70 attachement on yellowtail kingfish.

2. Methods

2.1 Animal Ethics

This trial conducted was using yellowtail kingfish (*Seriola lalandi*) with all animal husbandry and experimentation methods approved by the Curtin University Animal Ethics Committee (ARE2017-14).

2.2 Treatment diets

A total of 5 diets were used throughout a preceding growth study (not reported here) and the current fluke challenge trial. A total of 100 kg of 3mm commercial yellowtail kingfish pellets were
80 ground into a powder and the various immunostimulants were added to 12 kg batches. The immunostimulants used were Natural garlic powder (Spencers) at two dietary inclusion levels (10 and 20 g/kg) and two commercial garlic derivatives (labelled A and B), included at the manufacturer's recommended dose of 2 g/kg and the control diet with no garlic additives. The fish were maintained on the different diets for a total of 9 weeks (4 week grow out trial, 2 weeks holding at ACAAR and 2 week acclimation at CARL and 1 week post fluke challenge).

2.3 YTK acclimation and water quality

A total of 120 yellowtail kingfish (*Seriola lalandi*) were sourced from The Australian Centre for Applied Aquaculture Research (ACAAR). The yellowtail kingfish were transported to the Curtin Aquatic Research Laboratory (CARL) after a one month long immune stimulant growth trial, where
90 the fish were fed prescribed diets of various garlic supplemented pellets.

The fish were acclimated in 15 independent recirculating aquaculture tanks at CARL for 2 weeks prior to fluke exposure. During this time fish were fed approximately 2% of their body weight daily (on average 22 g/day/tank). Between 7 and 10 fish were initially stocked into each tank at a stocking density of 2.9 ± 0.3 kg/m^3. Equal numbers were not stocked into each tank because fish had individual tags and keeping them in similar groups made the sorting process more streamlined. All seawater used was filtered and UV sterilised to reduce risk of infections and contamination and water quality was maintained by regular 50% water exchanges. Water temperature was maintained at 21.9 ± 2.5 ^{o}C and salinity at 37 ± 2 ppt. Water quality (ammonia, nitrite and nitrate) was measured daily.

100

2.4 Host challenge

Neobenedenia girellae eggs were sourced from James Cook University in Townsville, Queensland. Approximately 5000 eggs where sent via express airfreight to the Department of Primary Industries and Regional Development, South Perth. Eggs were incubated in the dark in Petri dishes at ambient temperature (22 ± 0.5 °C) for 5 days and 75% of the water in each dish was exchanged daily with freshly oxygenated sterile seawater.

The fluke challenge began when >90% of the oncomiracidia had hatched in the Petri dishes. The fluke to fish ratio was pre-set at 30 oncomiracidia per fish, meaning each fish could potentially have 30 oncomiracidia attached. At the start of challenge, dissolved oxygen (DO) levels in tanks were
110 increased to between 20-25 mg/L with supplemented oxygen and all air and water flow were stopped for 3 hours during the inoculation period to allow the oncomiracidia to attach to a host before being drawn into the filter. The time of initial inoculation was recorded and DO levels were monitored at regular 30 minute intervals for 3 hours. At the end of the 3 hours all water and airflow was turned on and Petri dishes removed from each tank.

Attached oncomiracidia were allowed to grow for six days post challenge. After this six days fish were bathed in freshwater to dislodge the parasites as per Militz et al. (2013). The freshwater was then filtered through an 80 micron sieve and samples were collected and preserved in 10% formalin prior to enumeration.

2.5 Data analysis

120 A permutation multivariate analysis of variance (PERMANOVA) was undertaken to analyse the recruitment rate of oncomiracidia per fish. The decision to carry out a PERMANOVA analysis in place of a one-way ANOVA was due to the data not being normally distributed and data transformations did not improve the normality. All analysis was undertaken on PRIMER-7 and PERMANOVA+ statistical package. Graphs were produced on Microsoft Excel for MAC 2011. All statistical significance was accepted at $p<0.05$.

3. Results

3.1 Fluke attachment

The addition of the various garlic products to the diets did not yield significantly different rates of

130 *Neobenedenia girellae* oncomiracidia attachment on *Seriola lalandi* (P(perm) = 0.4647) (fig. 1).

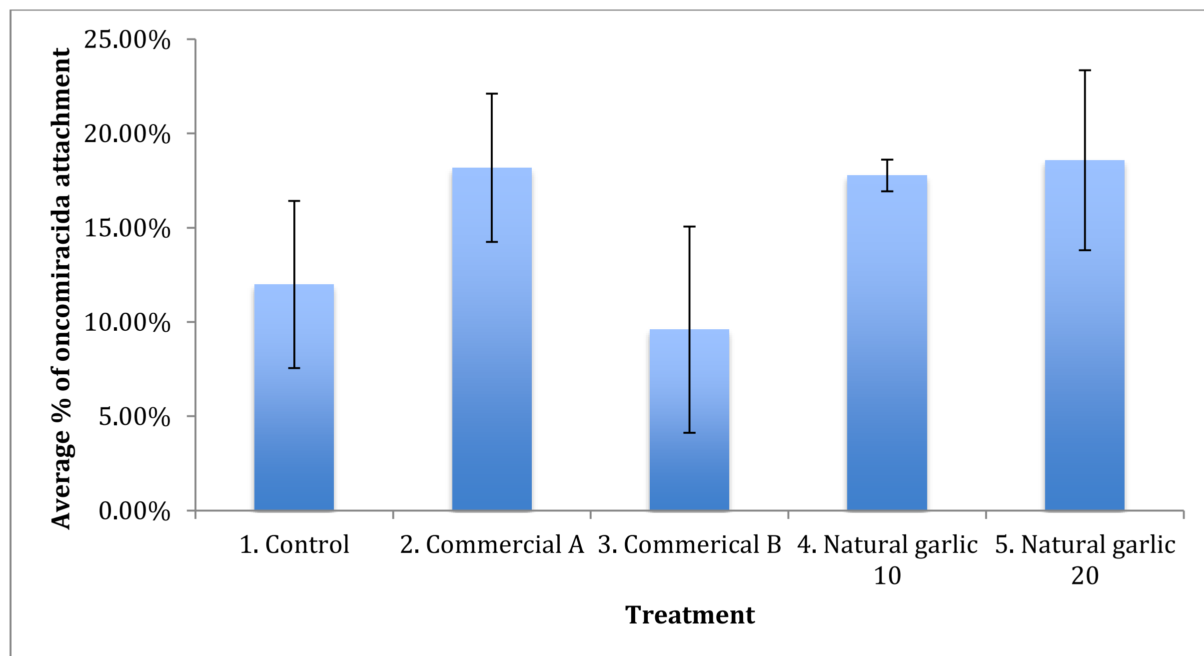

Figure 1: Mean percentage of oncomiracidia attachment (± SE) of *Neobenedenia girellae* on *Seriola lalandi* fed 1. Control, 2. Commercial A (2g/kg), 3. Commercial B (2g/kg), 4. Natural garlic (Spencers) 10g/kg (N10) and 5. Natural garlic (Spencers) 20g/kg (N20).

After an 8 week conditioning period, the addition of commercial B synthetic garlic supplement yielded a slight decrease on oncomiracidia attachment of 9.6± 5.5% (mean% ± SE) compared to the control diet of 12.0 ± 4.4%. The greatest difference between treatment groups was between the control and natural garlic high diets, where mean oncomiracidia attachment rates increased from 12.0 ± 4.4% to 18.6 ± 4.8%. Interestingly, the mean oncomiracidia attachment percentage of

140 treatment group N20 was higher than N10 at 18.6 ± 4.8% and 17.8 ± 0.8%, respectively. Commercial A product yielded 18.2 ± 3.9% oncomiracidia attachment rate, which an increase from the control treatment, suggesting no immunostimulant effect on the fish. No significance was reported between each treatment group.

4. Discussion

Feeding commercial B synthetic garlic at 2 g/kg very slightly reduced the number of *N. girellae* oncomiracidia attaching to yellowtail kingfish compared to the control diet. All other garlic supplemented diets yielded higher percentages of *N. girellae* attachment than the control. This study did not see a reduction of oncomiracidia attachment in any of the garlic supplemented
150 treatments, in fact there was an increase. Both natural garlic treatments (N20 and N10), yielded high oncomiracidia attachment percentage rates compared to the control group. The N20 treatment group had an overall higher percentage of oncomiracidia attachment compared to N10, suggesting a high concentration of garlic does not reduce oncomiracidia attachment. This finding is supported by Militz et al. (2013) study on infection success of *N. girellae* on saltwater barramundi, where their high garlic concentration yielded greater oncomiracidia attachment.

Comparing *N. girellae* infection success in this study where infection percentage ranged from 9.59-19.58% to Militz et al. (2013) where the infection percentage ranged from 22-25% after a 10 day conditioning period and 6-19% after 30 day conditioning period. In this study oncomiracidia infection success was in the range of 9.6- 18.6 %. Therefore, the infection success percentage in this trial is
160 therefore comparable to that of Militz et al (2013), demonstrating that our methods of infection were successful.

Interestingly, this challenge has shown higher concentrations of natural garlic added into feed diets are not necessarily beneficial. There was no significant difference between N10 and N20 treatments, however on average N20 had more attachment success of *N. girellae* compared to N10, at 18.85±4.78% and 17.88±0.84% respectively. This was found to be true with Militz et al. (2013) and their study on infection success of *N. girellae* on previously uninfected saltwater barramundi (*Lates calcarifer*). It was found after 30 days post supplemented feed of 150 mL/kg and 50 mL/kg garlic extract, infection prevalence was at 64% and 50% respectively (Militz et al., 2013). Why a higher concentration of garlic supplemented into feed pellets does not have a greater impact on the
170 reduction of oncomiracidia attachment is not fully understood to date.

To compare the concentrations of allicin in garlic used between Militz et al. (2013) and this current study. Militz et al. (2013) found the concentration of allicin present in their garlic liquid extract is 0.68 µL/L and dried natural garlic contains between 7737.0 – 9498.2 µg/g of allicin, which is dependent on the temperatures at which garlic was dried at (Ratti et al., 2007). Lower drying temperatures retains more active allicin in dried garlic powder (Ratti et al., 2007). Knowing that dried garlic has a high concentration of allicin compared the garlic extract, a lower dose was used to

make both natural garlic treatments for this study. However, even at lower garlic doses for N10 and N20 treatments, the allicin content was higher compared to Militz et al. (2013) dosages in their treatments. This could explain the higher oncomiracidia attachment rates in this trial however, there
180 further investigation is needed to determine the true effect of allicin on yellowtail kingfish immune system.

Other studies have shown a significant reduction of monogenean infection on tropical freshwater guppys (*Poecilia reticulata*) a well known home aquaria fish, when fed on a diet of garlic supplemented (10 and 20%) when compared to the control diet (Fridman et al., 2014). Once again the results of the Fridman et al. (2014) study were very different to this study, as this study found no effect of the garlic additives on oncomiracidia attachment. This could be explained by mucous make up and fish metabolism, yellowtail kingfish are high energy and would have different mucous make up which can affect the effectiveness of the anti-parasitic compounds of garlic.

Garlic is a known immunostimulant however, it is unknown exactly how garlic affects the immune
190 system (Militz et al., 2013). It has been shown that one of the innate defence mechanisms of fish against parasites and bacteria is to over produce the mucous cells as this helps the facilitation of other immune defences to reach the surface of the body (Fridman et al., 2014; Ghehdarijani et al., 2016). One study on freshwater guppy (*Poecilia reticulate* (Peters)) and monogenean (*Gyrodactylus turnbulli*) found that orally feeding garlic supplemented diets to fish significantly reduced the prevalence of parasite attachment (Fridman et al., 2014), which indicates a positive immune response against monogeneans. Studies on a freshwater carp (*Laheo rohita*) and rainbow trout (*Oncorhynchus mykiss*) found an increase in leucocytes after being fed garlic supplemented diets (Nya and Austin, 2009; Sahu et al., 2007). However, in this study the addition of garlic supplements in diets did not significantly decrease number of parasite attachment. Further investigation
200 questioning the attachment success is needed to ascertain reasons why this were the case and test for potential confounding factors.

Garlic is known for its beneficial properties on the cardiovascular and immune system (Harris et al., 2001). Increasing lysozyme activity which is associated with the innate immunity of fishes (Militz et al., 2013). Innate immunity is defined as rapid defence that utilises proteins and gene coding for identification of potential harmful substances (Fearon and Locksley, 1996; Magnadóttir, 2006). Although innate immunity is the fundamental defence mechanism used by fish, temperature, handling stress and overstocking suppresses the efficiency of innate immunity (Magnadóttir, 2006). With the combination of fluctuating temperatures (21.9 ± 2.5°C), sometimes within a 24 hour

period, this may affect the fish immunity. Which could explain higher oncomiracidia attachment
210 success for garlic-supplemented feeds of commercial A and B, N10 and N20 compared to the control diet, rather than a decrease in oncomiracidia attachment as was expected.

The different physiological needs of yellowtail kingfish compared to barramundi and freshwater guppy potentially explain confounding results (Fridman et al., 2014; Militz et al., 2014, 2013). Militz et al. (2013), methodology had one 12.4 ± 0.4 g fish in each 15L tank (stocking density of 0.82 kg/m^3) barramundi in a 15L RAS aquaria compared to the higher stocking density of 2.8 ± 0.3 kg/m^3 used in this trial. High stocking densities are known to cause stress on fish and reduce water quality affecting over all immunity of fish and increasing susceptibility to secondary diseases and infections (Lafferty et al., 2015).

The physiology of yellowtail kingfish is different from other similar studies undertaken with
220 barramundi, freshwater carp and rainbow trout (Militz et al., 2013; Nya and Austin, 2009; Sahu et al., 2007). Yellowtail kingfish are highly active with high energy demands compared to barramundi that are relatively sedentary and with low oxygen needs (Glencross and Felsing, 2006; Pirozzi and Booth, 2009). Yellowtail kingfish being highly active and constantly swimming around the tank, oncomiracidia attachment rates within tanks varied a great deal (between 0 and 19). The variation of attachment success would be explained by the fish activity and higher metabolism rate breaking down the active ingredient of garlic and therefore not being excreted through the mucous. A higher concentration of natural garlic compound can be trialled in the future to test whether or not yellowtail kingfish metabolism has any effect.

Water temperature and salinity levels also affect oncomiracidia infection rates. Brazenor and Hutson
230 (2015) found that cooler temperatures (22°C) and hypersaline (40ppt) conditions increased oncomiracidia infection rates and longevity on salt-water cultured barramundi. During this trial salinity varied between 35-40ppt and temperatures averaged 21.9°C. Suggesting the cooler temperatures and high salinity in this trial may have attributed to the increased oncomiracidia attachment rates of the control treatment group. However, the need for further investigation of factors that affected infection rate and success is needed to fully understand and determine.

In conclusion, all garlic supplemented treatment groups except for commercial B saw an increase in *N. girellae* attachment success compared to the control group. Despite there being no significant differences between treatments, there was an increase in fluke attachment in all other garlic supplemented diets. This could be explained by multiple factors such as, fish stress, variation in daily
240 temperatures and high salinity water conditions favouring the development of *N. girellae*

oncomiracidia, as stated in Brazenor and Hutson, (2015). Further studies, are suggested to measure or better control those factors mentioned to determine if these factors did attribute to the results obtained.

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Anthelmintic herbal extracts as potential prophylactics or treatments for monogenean infections in cultured yellowtail kingfish (*Seriola lalandi*)

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Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institution.

Jack Ingelbrecht

Abstract

Monogenean ectoparasites pose a considerable threat to the aquaculture of yellowtail kingfish (YTK), *Seriola lalandi*. Current chemotherapeutic or freshwater bathing treatments for these parasites can stress fish (resulting in decreased immunocompetence), are expensive, and can be labour intensive. Medicinal herbs, many of which possess various anti-parasitic properties, show promise as an alternative or supplement to current treatments. Potential anthelminthic effects of garlic, rosemary, and commercially manufactured herbal products were trialed both *in vitro* and *in vivo* on two species of monogeneans, *Neobenedenia girellae* and *Zeuxapta seriolae,* to assess their feasibility for use in yellowtail kingfish aquaculture. The survival of adult *Neobenedenia girellae* and *Zeuxapta seriolae* and *N. girellae* oncomiracidia larvae were assessed *in vitro*, by exposing parasites to 5 mL of seawater solutions containing various herbal products. All herbal-seawater solutions significantly reduced the survival of mature *N. girellae* and *Z. seriolae* and *N. girellae* oncomiracidia relative to the control. Following this *in vitro* trial, I conducted two *in vivo* trials examining the preventative and curative effects of rosemary and garlic on *Zeuxapta seriolae* infections, by supplementing these products into YTK feed. Preventative diets were fed to YTK for 30 days prior to challenging fish with *Z. seriolae* oncomiracidia while curative treatment diets were fed to YTK already infected with *Z. seriolae* for 10 and 20 days. I also examined the effect of these curative treatment diets on the recruitment of juvenile *Z. seriolae*. Supplementation of rosemary and garlic products into YTK feed 30 days prior to parasitic challenge significantly reduced *Z. seriolae* infection success, while supplementation of garlic powder, rosemary extract, and rosemary oil onto pellets significantly reduced *Z.*

seriolae abundance on YTK after 20 days of feeding on curative treatment diets. Supplementation of garlic powder and rosemary extract onto pellets also significantly reduced juvenile *Z. seriolae* recruitment.

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1 Introduction

1.1 Aquaculture in Australia

Global aquaculture has grown continuously over the last 4 decades, producing 101.1 million tons of fish and aquatic plants in 2014 (FAO, 2016). Expansion of the aquaculture industry is a consequence of an increasing global demand for seafood, which in people has been linked with improved heart health (due to high contents of omega-3 fatty acids), and a decrease in supply from wild capture fisheries resulting in declines in wild fish stocks (Domingo, 2014; Grealis et al., 2017). In 2014, aquaculture accounted for approximately 44.1% of the total global seafood production, and is the fastest growing food-producing sector in the world (FAO, 2016). Given that world resources are limited with respect to terrestrial protein production for human consumption, aquaculture is now considered a more important sector than capture fisheries in which to invest government resources for seafood protein (Nadarajah & Flaaten, 2017). Production of a kilogram of protein via aquaculture also has a much smaller carbon footprint than producing a kilogram of protein in current terrestrial animal production industries (Torrissen et al., 2011).

Australian aquaculture follows the global trend in being one of the fastest-growing primary industries in the country, with a production value that has been increasing annually by approximately 6% since 2005 (ABARES, 2017). In 2015-2016, the aquaculture industry accounted for 43% of Australia's total

fisheries production (ABARES, 2017), making it Australia's fifth most valuable food industry (FRDC, 2015).

The earliest example of aquaculture in Australia is the culture of shellfish in the 1860s, with salmonids later being introduced in 1864 (Haward, 2016). Currently, the Australian aquaculture sector encompasses a diverse range of species, with products varying across states and territories (Mazur & Curtis, 2006; Haward, 2016). The largest producer is Tasmania, which, in 2012-13, accounted for 58% of the country's total production (Stephan & Hobsbawn, 2013). Australian aquaculture products are dominated by five major groups of species: salmonids, pearl oysters, tuna, edible oysters, and prawns (ABARES, 2017). These five encompass approximately 91% of the gross production value of Australian aquaculture (Table 1.1) (ABARES, 2017). Atlantic salmon are the most farmed species in Australia, with the production volume increasing by 168% (up by 35,335 tons) over the last decade (ABARES, 2017).

Table 1.1 Volume (in tons) and Gross Value of Production (GVP) for the five major farmed species in Australian aquaculture in 2015-16.

Species	**Volumes (tonnes)**	**GVP (AU$ millions)**
Salmon	56,319	717.7
Tuna	8,895	126.9
Edible oysters	11,345	97.0
Pearl oysters	Not available	78.4
Prawns	4,628	86.5

1.2 Yellowtail kingfish (*Seriola lalandi*) aquaculture

Yellowtail kingfish, *Seriola lalandi*, is a species of marine, pelagic, piscivorous finfish, distributed globally in subtropical and temperate latitudes (Moran et al., 2007; Bowyer et al., 2012). In Japan, the production of kingfish accounts for an estimated 70% of total finfish aquaculture production (Poortenaar et al., 2001). The yellowtail kingfish aquaculture sector in Japan is almost totally reliant on the capture and growth of wild juveniles in sea cages (Poortenaar et al., 2001). While commercial production of yellowtail kingfish in Japan is relatively small compared to other *Seriola* species, specifically Japanese amberjack (*Seriola quinqueradiata*) and greater amberjack (*Seriola dumerilli*), total production is still far higher than any other country (Nakada, 2002; Moran et al., 2007).

Yellowtail kingfish has been identified as a potentially valuable commercial aquaculture species due to high growth performance, and adaptability to sea-cage conditions (Poortenaar et al., 2001; Fowler et al., 2003; Stuart & Drawbridge, 2012). The majority of yellowtail kingfish aquaculture is concentrated in the cool-temperate waters of South Australia, where it is the second largest (and most rapidly growing) aquaculture sector after southern bluefin tuna (Tanner & Fernandes, 2010). Commercial production of yellowtail kingfish in South Australia was estimated at over 2000 tons in 2004-05 (Chambers & Ernst, 2005), with the industry in Australia now producing over 4000 tonnes per year (FRDC, 2013).

Recently, yellowtail kingfish production is being trialed in in Providence Bay in New South Wales and off Geraldton in Western Australia (Booth et al., 2010). Both regions are subjected to warmer waters than areas where yellowtail kingfish are produced in South Australia, favoring an increase in feeding activity and growth rate.

1.3 Parasites and diseases impacting yellowtail kingfish aquaculture

Morbidity and mortality associated with infectious disease is the most significant constraining factor on the production and growth of fish in aquaculture (Austin, 2012). Production losses and associated negative economic impacts due to pathogen infections can be as high as 50% (Assefa & Abunna, 2018). Mitigating the impacts of infectious disease is made increasingly difficult by the growing number of pathogens discovered in the industry, as well as the variation in susceptibility among different species of fish (Secombes & Wang, 2012). While morbidity and mortality rates are considered the most economically and ecologically important consequences of infectious disease in aquaculture (Ortega et al., 1995), secondary effects such as stunted growth and decreased feed conversion efficiency can also significantly decrease productivity.

In Japan there have been at least 26 documented diseases that affect commercially cultured *Seriola* spp. (Sheppard, 2004). Farming fish in sea cages typically involves stocking individuals at a higher density than would be found in wild populations (Hutson et al., 2007a). As a result, cultured fish potentially develop higher pathogen burdens than wild fish, as farming conditions in sea-

cages may be accompanied by a higher rate of disease transmission (Ogawa, 1996). Fish are more susceptible to disease if there is an increase in stressors such as handling, overcrowding, poor nutrition and poor water quality (Reed et al., 2002; Conte 2004). These factors also impact the fish's immune response to infections (Conte, 2004).

In the Australian yellowtail kingfish industry, parasites are the most important disease-causing agents affecting production (Ernst et al., 2002). Yellowtail kingfish juveniles for industry are produced in land-based hatcheries, and generally isolated from parasite infections through biosecurity measures (Hutson et al., 2007a). Once fingerlings are moved to sea cages, however, they are susceptible to parasites present in the local grow out environment (Hutson et al., 2007a). Wild fish are believed to be the primary source of infection in sea-cage aquaculture (McVicar, 1997), and therefore parasite transfer likely occurs when infected wild individuals pass within close proximity to sea-cages (Hutson et al., 2007a).

Forty-two parasite taxa have been reported to infect wild yellowtail kingfish (Ernst et al., 2002; Sharp et al., 2004). Of these, three species (all monogeneans; *Benedenia seriolae, Neobenedenia girellae* and *Zeuxapta seriolae*) are the most significant primary pathogens in cultured yellowtail kingfish and require considerable management (Sharp et al., 2003; Hutson et al., 2007b).

Monogeneans are ectoparasitic flatworms (commonly referred to as flukes), which infect a wide range of fish and aquatic invertebrates in marine and

freshwater ecosystems worldwide (Reed et al., 2002; Whittington et al., 2011). Monogeneans are often highly host-specific (Whittington et al., 2000), and the pathology of infections can vary dramatically between different families of these parasites and the hosts they infect. Species that infect only the gills of their fish host (e.g. *Zeuxapta seriolae*) cause pathology through direct feeding on gill epithelia and blood, which can inhibit respiratory gas exchange (Thoney & Hargis, 1991). Taxa which infect primarily the skin of their fish host (e.g. *B. seriolae* or *N. girellae*) generally feed on epithelial tissue, which can result in secondary infections brought on by open wounds (Egusa, 1983; Thoney & Hargis, 1991). Host response to monogenean infections can involve the infiltration of inflammatory cells and destruction of localized tissue (Kim & Choi 1998).

Monogeneans have a direct, single host life cycle, where fertilised eggs are shed directly into the water and the resulting ciliated larval parasite (oncomiricidium) hatches and swims freely to infect another suitable host; because of this, they can proliferate rapidly in aquaculture systems (Mooney et al., 2008). Once a host is located, the larva crawls along the host's body to its preferred location (usually gills or skin) (Reed et al., 2002). Eggs shed by sexually mature monogeneans can become trapped in the netting of sea-cages or gills of fish, meaning they are retained in the aquaculture system and therefore present a high risk of reinfection (Williams, 2010). Furthermore, monogenean eggs are resistant to treatments, meaning that repeated treatments that target larvae and adults are required to eradicate infections (Reed et al., 2002). Egg hatching and adult developmental times to sexual maturity are primarily dependent on water

temperature, which can vary from days in temperate to subtropical waters, to 5-6 months in polar waters (Reed et al., 2002).

Neobenedenia girellae and *Benedenia seriolae* are monopisthocotylean (single attachment haptor) monogeneans of the family Capsalidae, which inhabit external surfaces of skin and fins of their fish hosts (Sharp et al., 2004; Chambers & Ernst, 2005). Following successful attachment of the oncomiricidia, the parasites feed on mucous and epithelial cells (Reed et al., 2002; Sharp et al., 2004). Fish infected by these flukes may exhibit flashing, whereby the infected individual will rub the infected areas on the bottom and sides of their enclosure in an attempt to reduce irritation (Egusa 1983). This flashing behavior can lead to external injuries, which in turn can lead to secondary infections and mortality (Sharp et al., 2004).

Zeuxapta seriolae is a polypisthocotylean (many attachment haptors) monogenean species of the family Heteraxinidae, which inhabits the gill lamellae of infected fish via haptoral clamps and primarily grazes on the host's blood (Mooney et al., 2006). Infections with *Z. seriolae* are often difficult to identify in culture settings, as the gills cannot by closely inspected without directly handling the host (Sharp et al., 2004). Signs of *Z seriolae* infections include increased operculum movement, increased mucous production, reduced appetite, and a loss of equilibrium (Sharp et al., 2004; Mooney et al., 2006). Heavy infections with *Z. seriolae* can result in severe anemia and subsequent mortality (Sharp et al., 2004; Mooney et al., 2006).

1.4 Treatments for monogenean infections

Treatments for monogeneans can be classified as prophylactic (preventing parasites from infecting a host) or curative (removing parasites after they have infected a host). A prophylactic treatment administered as part of a routine diet in aquaculture systems is a practical solution to managing monogenean infections as no handling of livestock is required, the treatment is delivered directly to the host (rather than to the hosts environment), and the stresses caused by heavy parasite burdens are avoided (Dunn et al., 1990; Militz et al., 2013a). Despite the potential advantages of prophylactic treatments over the curative means of managing monogenean infections (i.e. the avoidance of stresses and mortalities associated with parasite burdens), few are used in aquaculture due to the compromised practical applications of current synthetic anthelmintics (i.e. praziquantel), such as host toxicity, and low palatability (Williams et al., 2007; Militz et al., 2013a; Partridge et al., 2014).

Chemical treatments administered after monogenean infections have been diagnosed is the current standard for managing these parasites in aquaculture. Administration techniques typically include baths or oral administration (Zoral et al., 2017). The type of treatment used depends on a variety of factors such as the species of parasite, and logistics of the facility where treatment is being administered (Eiras et al., 2008).

Bathing treatments for monogenean infections involves immersing infected fish in a solution of the chosen chemical in order to dislodge or kill attached

parasites. In sea-cage culture, this can be done by enclosing the cage in materials, such as tarpaulin, in order to cut it off from the external environment, or by removing fish from sea-cages and placing them in a container with the treatment solution. Research shows that any handling of fish in the bathing process can result in significant stress to the fish (potentially more so than the direct effects of the chemotherapeutics used in treatments) (Costello, 1993). Furthermore, bathing treatments are often costly, labor-intensive and time consuming, especially if fish are removed from holding tanks/cages in the process, meaning that it may be days before all infected fish have been treated (Costello, 1993; Parsons et al., 2001).

Common bath treatments include freshwater or hypo-saline water (for marine fish) and saltwater (used to treat freshwater fish) and are advantageous in not being restricted by regulated drug withholding periods (Eiras et al., 2008). However, these hypo- and hyper- saline bath treatments are less effective against gill infecting monogeneans due to their ability to shield themselves between the lamellae and mucus layers, require access to adequate fresh/saltwater sources, and bathing times are often lengthy compared to chemical treatments (Thoney & Hagris, 1991; Parsons et al., 2001).

Chemotherapeutic baths generally involve the use of chemicals such copper sulfate, hydrogen peroxide, potassium permanganate and trichlorfon (Zoral et al., 2017). These can be highly effective at killing adult monogeneans that inhabit both the skin and gills of their host, with the bathing period less time consuming in contrast to fresh/saltwater baths (Schmahl, 1991; Ernst et al., 2005).

However, chemical bath treatments are expensive, potentially dangerous to the user in concentrated forms, harmful to fish when incorrectly administered, can adversely impact water quality, and can leave toxic residues in fish flesh (Ernst et al., 2002; Sharp et al., 2004; Hoque et al., 2016). A method commonly used in Australia for treating yellowtail kingfish infected with monogeneans is bathing individuals in hydrogen peroxide. While this treatment is effective for mature and immature monogeneans on their fish host, reinfection can occur immediately as un-hatched eggs are resistant to this treatment (Sharp et al., 2004; Ernst et al., 2005).

Oral administration of anthelmintics through direct incorporation of the treatment into the feed is considered far easier to apply to fish in sea-cages than bath treatments (Costello, 1993). Oral treatments typically require less manual labour and are less stressful to fish (as there is no need for physical handling) (Costello, 1993). Fish in sea-cages can also be treated more rapidly, thereby reducing the chances of reinfection from nearby untreated fish in other sea-cages, whilst allowing fish to maintain their usual feeding routine (Costello, 1993; Conte, 2004; Williams et al., 2007). Orally administered treatments are, however, not without disadvantages: only feeding fish will receive the treatment; and the dosage can become increasingly complicated if there is wide variation in size and consumption rates within sea-cages (Costello, 1993; Shet & Vaidya, 2013).

Examples of orally administered, synthetic anthelmintics used in aquaculture include praziquantel and mebendazole (Zoral et al., 2017), with the former used

to successfully treat the monogeneans *Z. seriolae* and *B. seriolae* in yellowtail kingfish (Williams et al., 2007). However, praziquantel is notably unpalatable to yellowtail kingfish and other *Seriola* spp. and rejection of feed pellets coated in this drug is a major impediment to successful delivery (Hirazawa et al., 2004; Williams et al., 2007; Partridge et al., 2014; Partridge et al., 2016). Palatability issues and other undesirable side effects of chemotherapeutic treatments have prompted efforts to explore less harmful alternative treatments to combat monogenean infections in aquaculture (Lee & Gao, 2012; Zoral et al., 2017).

1.5 Herbal extracts for anthelmintic use in aquaculture

Herbs and herbal extracts have been used in a variety of human and veterinary medicines, and are easily accessible throughout the world. Medicinal herbs contain known anti-pathogenic, anti-inflammatory and immunostimulant properties (Reverter et al., 2014; Zoral et al., 2017). The compounds derived from herbs and herbal extracts are often less expensive to produce than chemotherapeutic treatments, have been reported to leave far lower toxic residues in fish flesh, and have fewer adverse effects on water quality (Murthy & Kiran, 2013). Furthermore, some medicinal plants have been reported to stimulate appetite, and subsequently encourage weight gain in fish (Akrami, 2016). When consistently added to fish diets, medicinal plant products therefore have the potential to increase aquaculture productivity by increasing growth rates, and decreasing mortalities associated with illness. The potential use of herbal extracts (particularly garlic and rosemary) as prophylactic or curative treatments against monogenean infections is beginning to be explored in the

aquaculture of a wide range of aquatic species, due to the lack of the many negative side effects associated with chemotherapeutic treatments, low cost and ease of administration. However, their practical application and efficacy in preventing infections in yellowtail kingfish has yet to be determined.

Rosemary (*Rosmarinus officinalis*) belongs to the Lamiacaeae family, and is native to the Mediterranean region. Rosemary and associated extracts have been shown to possess anti-parasitic, anti-inflammatory, antibacterial, antithrombotic, antinociceptive and anticancer properties (Zimmermann, 1980; Petiwala & Johnson, 2015; Zoral et al., 2017). In aquaculture, extracts prepared from rosemary leaves have been used to successfully treat monogenean infections in carp (Zoral et al., 2017); however, there have been no published studies on the use of rosemary to treat monogenean infections in yellowtail kingfish. Of the major components in rosemary extracts, the 1,8-Cineole compound has been shown to have the strongest effect on monogeneans (Zoral et al., 2017). Furthermore, ethanol has been shown to extract higher concentrations of the 1,8-Cineole compound than distilled water extraction (Zoral et al., 2017).

Garlic (*Allium sativum*) belongs to the family Amaryllidaceae, and is native to Central Asia and northeastern Iran. Garlic has been shown to possess antimicrobial, anti-hypertensive, and anti-parasitic properties (Lee & Gao, 2012). Like other medicinal herbs, garlic has been reported to increase appetite, promote growth and enhance immune system functioning (Lee & Gao, 2012). The observed anti-microbial and immunostimulant properties associated with garlic derived products are attributed to the phytochemical allicin (diallyl thiosulfinate) and its derivatives (Nya et al., 2010; Militz et al., 2013a). Garlic

supplementation has been previously used in aquaculture, with a relatively short period (14 days in some studies) of supplementation required before the associated benefits (increase in immune-competency) are achieved (Sahu et al., 2007; Nya & Austin, 2009). Garlic extract has been applied as a therapeutic bath to treat *Neobenedenia* sp. in farmed barramundi (*Lates calcarifer*) and other commercially produced species (Sahu et al., 2007; Nya & Austin, 2009; Militz et al., 2013a; Militz et al., 2013b). Militz et al. (2013a) demonstrated that feed supplemented with garlic can significantly reduce the attachment success of *Neobenedenia* sp. in barramundi, when fed for 30 days prior to parasites being introduced into the system. Therefore, garlic may also have use as a prophylactic treatment for monogenean infections in yellowtail kingfish.

1.6 Aims and hypotheses

The aims of this study were to investigate whether rosemary and garlic extracts supplemented into the diet of yellowtail kingfish, could be viable prophylactic and/or curative treatments against monogenean infections in yellowtail kingfish aquaculture.

The hypotheses tested were:

1. Garlic and rosemary solutions will inhibit the survival of *Neobenedenia girellae* oncomiracidia and adult *N. girellae* and *Zeuxapta seriolae in vitro,* consistent with the anthelmintic properties described in Militz et al. (2013b) and Zoral et al. (2017) respectively.
2. Yellowtail kingfish fed a garlic supplemented diet will gain more weight and have a lower food-conversion ratio in relation to fish fed on a control

diet, as garlic has been shown to stimulate appetite and increase growth (Lee & Gao, 2012).

3. Garlic and rosemary supplemented diets will significantly inhibit the prevalence and abundance of *Zeuxapta seriolae* infections on yellowtail kingfish, when fed to fish as a preventative treatment.
4. Garlic and rosemary supplemented diets will significantly reduce *Zeuxapta seriolae* abundance on infected yellowtail kingfish when used as a curative treatment.

2 Materials and Methods

Animal ethics approval: R3038/18

2.1 Anthelmintic assessment of herbal extracts *in vitro*

Two herbal extracts and two commercially available products containing herbal extracts were selected for initial *in vitro* assessment of their anthelmintic properties against *Neobenedenia girellae* and *Zeuxapta seriolae*. The design for this *in vitro* trial included five treatment groups (four herbal extract solutions and a seawater control), with 30 individuals of each of the following parasite species and life stages exposed in triplicate in 6-well cell culture plates: adults and oncomiricidia of *N. girellae* and adult *Z. seriolae*.

The products tested in this trial were Spencers™ dry rosemary leaves, Spencers™ garlic powder, and the commercially manufactured Ridleys™ products Aquagarlic-A and Aquagarlic-P – both containing high amounts of allicin. Aquagarlic-P is labeled as an anti-parasitic product, while Aquagarlic-A is labeled as an anti-microbial product. Solutions for *in vitro* exposure were prepared by stirring a specific dose of the raw herbal materials/commercially manufactured herbal products (Table 2.1) with 100 mL seawater at 100^{0}C for ten minutes. The supernatants were then filtered through 2 µm syringe filter units, and stored at 4^0 C in 50 mL test tubes until use.

Table 2.1 Dosage of herbal ingredient stirred into 100 mL seawater for 10 minutes at 100^0 C for *in vitro* trials.

Herbal ingredient	Dose g/100 mL seawater
Dry rosemary leaves	20.0
Garlic powder	1.0
Aquagarlic-A	0.3
Aquagarlic-P	0.3

Newly hatched *N. girellae* oncomiracidia were isolated via pipette from eggs held in seawater in culture plates, counted and sorted into three wells each containing thirty oncomiracidia in 1 mL of seawater for each of the five treatments. Adult *N. girellae* and *Z. seriolae* were collected from the skin and gills of infected yellowtail kingfish and allocated to three wells of the 6-well culture plates with thirty individuals per well in 1 mL of seawater for each of the five treatments. Five milliliters of the allocated treatment solution were then added to wells and the parasites were monitored constantly for up to one hour using a light microscope, after which parasites were monitored once each hour. Death was judged by cessation of movement. The time to death was recorded at 100% mortality for each replicate. The time from the first parasitic mortality was also recorded to determine the range of mortalities.

2.2 *In vivo* trials incorporating anthelmintic herbal extracts into diet

Two *in vivo* dietary supplementation trials were conducted in order to assess the potential use of three of the herbal extracts used in the *in vitro* trials (i.e. Rosemary extract, Garlic extract and Ridley's Aquagarlic-P (trial 1 only)) as: 1) a

prophylactic treatment to reduce infection success of *Z. seriolae* and 2) as a curative treatment for existing infections with *Z. seriolae*. Trial 2 replaced the Aquagarlic-P herbal extract with a commercially manufactured rosemary extract (Range Products™ essential rosemary oil (30% 1,8-Cineole)).

2.2.1 *Trial 1 – Herbal extracts as prophylactic treatment for* Zeuxapta seriolae *infections*

The diets prepared for this *in vivo* trial consisted of three treatment diets and one un-medicated control diet (each diet being distributed among four tanks; i.e. four replicates per treatment). The three herbal products used to manufacture the three treatment diets were: Spencers™ dried rosemary leaves; Spencers™ garlic powder; and Ridleys™ Aquagarlic-P. The amount of herbal additives used in the making of 1 kg of each diet varied between treatments (Table 2.2), with the amount being based on either the recommendations of the manufacturer, or on previous experiments (Zoral et al., 2017). Each diet was prepared by first grinding Ridleys™ 3mm pelleted feed into a powder using a grinding mill, and mixing 1 kg of the powdered feed with 450 mL distilled water (281.5 mL distilled water for diet containing rosemary extract), and (for medicated diets only) with one of the three herbal products in a laboratory mixer for 10 minutes. The mixtures were then cut into 4.8 mm pellets, before being air-dried at 24^{0}C over three days. The un-medicated, control diet was prepared using the same methodology, without the addition of a herbal treatment. Moisture contents of all diets after the three-day drying period was determined to be less than 10%.

Zoral et al. (2017) demonstrated that 31 mg of 1,8-Cineole per 100 g of feed is enough to treat monogeneans in an aquaculture environment and that ethanol extraction (75% ethanol) is more effective at extracting the compound than aqueous extraction, with 100 g ethanol extract (made up of 10 g of dry rosemary and 50 mls of 75% ethanol) containing approximately 23 mg of 1,8-Cineole (Zoral et al., 2017). Dry rosemary leaves were purchased from Spencers™ in March 2018, and stored at 4^0C until use. Ethanol extraction was used to prepare rosemary extract to be mixed with yellowtail kingfish diet as per Zoral et al. (2017). This involved homogenising 10 g of leaves per 50 mL of 75% ethanol in a laboratory blender at room temperature, forming a fine suspension. The resulting suspension was transferred to a 50 mL plastic tube and centrifuged at 2890 x g (3900 rpm) for ten minutes at 21^0C. Supernatants were carefully collected by pipette and filtered through a 220-µm filter paper, with filtrates stored at -20^0C until use. The weight of 1 mL of ethanol extract was 0.8 g.

Table 2.2 Dosage of herbal ingredient mixed with 1 kg powdered yellowtail kingfish feed and 450 mL distilled water (281.5 mL distilled water in diet containing rosemary-ethanol extract) in preparation of *in vivo* preventative treatment diets. Dosages based on manufacturer recommendations or previous studies (Zoral et al., 2017).

Herbal ingredient	Amount per 1 kg powdered feed
Rosemary extract	168.5 mL
Garlic powder	10.0 g
Aquagarlic-P	3.0 g

One hundred and sixty juvenile yellowtail kingfish (mean weight 148 g) were obtained from the Australian Centre for Applied Aquaculture Research hatchery in Fremantle, Western Australia. Fish were transferred to the Indian Ocean Marine Research Centre's dedicated quarantine experimental room in Watermans Bay, Western Australia. Individuals were randomly distributed among twenty 300 L tanks (8 fish per tank), with each tank constantly receiving 180 L/hour of new, filtered sea water (>80% oxygen saturation) (average temperature = 21.9^{0}C), until the experiment started (three day acclimation period), and throughout the experiment. During the acclimation period, fish were fed un-medicated Ridley's 3 mm pellets, with fish being fed to satiety once per day. One day prior to commencing the trial, fish were individually weighed, and their weights recorded. Fish were anaesthetized using AQUI-S prior to being weighed, in order to minimize movement on scales and stress to fish during the handling process. After being weighed, fish were gently placed in an aerated recovery tank, before being returned to their allocated experimental tank.

Fish were fed the experimental treatment diets for 30 days, with each tank being allocated a fixed ration of 50 g experimental diet per day at the start of the experiment, with feed ration being increased by 5 g each week. Fish were fed at 09:00 hours each day, and again at 15:00 hours if fish had not consumed their full ration during the first feeding time. All feed going into each tank was weighed and recorded. Any pellets that were not consumed were removed 5 min after feed was first administered.

At the end of the 30-day trial, fish were individually weighed and the feed-conversion ratio calculated as the ratio of food consumed to biomass gained. Following the weighing of fish and fish being returned to their respective tanks, all incoming water into each tank was halted for two hours, and 400 *Zeuxapta seriolae* oncomiracidia obtained from eggs held in sterile-filtered seawater culture wells were added to each tank. Water flow was halted in order to give the oncomiracidia time to attach to a host and to avoid being washed out of the flow-through research tanks. After two hours, water flow was returned to 180 L/hr. Fish were fed to satiety using commercial yellowtail kingfish feed, for a further 14 days. The 14 day period was chosen so attached parasites would be able to grow to a size that they would be easily visible for macroscopic counting, but not sexually mature and reproducing within the experimental tanks. After this 14-day period, each fish was euthanized via an overdose of AQUI-S, before being placed in a bath containing praziquantel, in order to remove any attached parasites. Removed parasites from each individual fish were sieved out and fixed in 70% ethanol for subsequent counting.

2.2.2 Trial 2 – Herbal extracts as potential curative treatment for Zeuxapta seriolae *infections*

Treatment diets for Trial 2 were prepared by mixing 1 kg of Ridleys™ 9mm pellets with 15 mL fish oil and one of three herbal treatments (Table 2.3; rosemary-ethanol extract, Spencers™ garlic powder, or Range Products™ essential rosemary oil), in a cement mixer for 10 minutes, in order to coat the various herbal treatments onto the surface of the pellets. Pellets were then air

dried at 24^{0}C for one day. An un-medicated, control diet was prepared using the same methodology with the exception of adding a herbal treatment.

Table 2.3 Dosage of herbal ingredient mixed with 1 kg 9 mm pellets in preparation of *in vivo* curative treatment diets. Dosages based on manufacturer recommendations or previous studies (Zoral et al., 2017).

Herbal ingredient	Dosage per 1 kg 9 mm pellets
Rosemary-ethanol extract	168.5 mL
Essential rosemary oil	0.1 mL
Garlic powder	10.0 g

One hundred and forty four yellowtail kingfish (mean weight 700g) held in sea-cages that were naturally infected by *Zeuxapta seriolae* were transported to the Batavia Coast Maritime Institute in Geraldton, Western Australia. They were evenly split among twelve, 4000L research tanks (12 fish per tank). Each tank received 1200 L of new, filtered seawater per hour (>80% oxygen saturation) (average temperature = 21.5^{0}C) during the acclimation period (three days) and throughout the experiment. During the acclimation period, fish were fed Ridley's 9 mm pellets, with fish being fed to satiety once per day. One day prior to the start of Trial 2, ten yellowtail kingfish were sourced from the same sea-cage as these experimental fish. These fish were euthanized and bathed in praziquantel, in order to remove any attached monogeneans. All removed parasites were sieved out and counted using a light microscope, to estimate the mean number of attached parasites per fish at the start of the trial.

Fish were fed treatment diets (three supplemented and one control diet) to satiety twice a day for 20 days. Any pellets that were not consumed were

removed 5 min after feed was first administered. At day 10 of the trial, 50% of the fish from each tank were euthanized and bathed in praziquantel, in order to remove any attached parasites, with the numbers of removed monogeneans recorded. At the end of the 20-day trial, the remaining fish were euthanized and bathed in praziquantel, with any removed monogeneans being recorded. *Z. seriolae* were categorized and counted based on maturity as either juvenile or mature, using criteria including size, development of clamps, and shade.

2.3 Statistical analyses

Data were analyzed using JMP v10 (SAS Institute, Carey, NC) and R v6.1.15 (R Core Team, 2015). Differences in survival time of parasites among *in vitro* treatments were compared by one-way ANOVA, followed by Tukey's HSD test for post-hoc multiple comparisons. Kaplan-Meier survival analysis was not used because all parasites exposed to herbal treatments died within the time period of the trial. Residuals from the ANOVA were normally distributed.

Differences in starting weight, absolute weight gain and feed conversion ratio among fish fed different preventative treatment diets in the first *in vivo* trial were analyzed with a general linear model, with replicate tanks nested within treatments as a random effect. Residuals from the model were normally distributed. Where a significant effect of treatment was found, Tukey's HSD test was used for post-hoc multiple comparisons.

Differences in abundance of parasites among treatment diets in both *in vivo* trials were analyzed using a generalized linear mixed model, with abundance modelled as a negative binomial variable with a log link, and replicate tanks nested within treatments as a random effect. The residual by predicted plot showed a random scatter, with no discernible pattern. Where a significant effect of treatment was found, pairwise contrasts were made among all treatments, using a Bonferroni correction for an experiment-wide Type I error of 0.05.

3 Results

3.1 Anthelmintic assessment of herbal extracts *in vitro*

Time to death for each of the experimental groups is shown in Table 3.1. Due to insufficient numbers of mature *N. girellae*, mature *Neobenedenia* were exposed to the rosemary solution only. The survival time of mature *N. girellae* was significantly reduced by the rosemary solution compared to the seawater control ($F_{1,4} = 102.4$, $P < 0.0001$). The survival time of *N. girellae* oncomiracidia differed significantly among treatments ($F_{4,10} = 380.9$, $P < 0.0001$), with multiple comparison tests showing that all herbal treatments reduced survival time relative to the control, but there were no significant differences in survival time among the herbal treatments themselves. There was also a significant effect of treatment on the survival time of mature *Z. seriolae* ($F_{4,10} = 6.4 \times 10^5$, $p < 0.001$), with all herbal treatment reducing survival time relative to the control, and survival time least in the garlic powder and rosemary solutions, followed by Aquagarlic-A and Aquagarlic-P (Figure 3.1).

Table 3.1 *in vitro* effects of various herbal solutions on the survival of *Neobenedenia girellae* oncomiracidia (*Neobenedenia* O), mature *Neobenedenia girellae* (*Neobenedenia* M)(rosemary only), and mature *Zeuxapta seriolae* (n = 3 replicates; 30 parasites per replicate)

Treatment	Parasite spp.	Time to 100% mortality (min)	Range of Survival Time (min)
Seawater control	*Neobenedenia* (O)	545.20 ± 27.41	150.38 – 599.63
	Neobenedenia (M)	> 2160	300 - >2160
	Zeuxapta	> 2160	780 - >2160
Rosemary	*Neobenedenia* (O)	7.22 ± 0.40	3.53 – 7.98
	Neobenedenia (M)	53.25 ± 1.58	18.73 – 59.09
	Zeuxapta	42.10 ± 1.03	35.33 – 43.85
Garlic powder	*Neobenedenia* (O)	3.43 ± 0.19	1.98 – 3.78
	Zeuxapta	39.92 ± 1.15	26.97 – 41.37
Aquagarlic-A	*Neobenedenia* (O)	3.22 ± 0.23	1.16 – 3.68
	Zeuxapta	47.77 ± 1.71	38.95 – 51.05
Aquagarlic-P	*Neobenedenia* (O)	25.03 ± 1.36	15.48 – 27.30
	Zeuxapta	57.3 ± 1.30	35.23 – 59.5

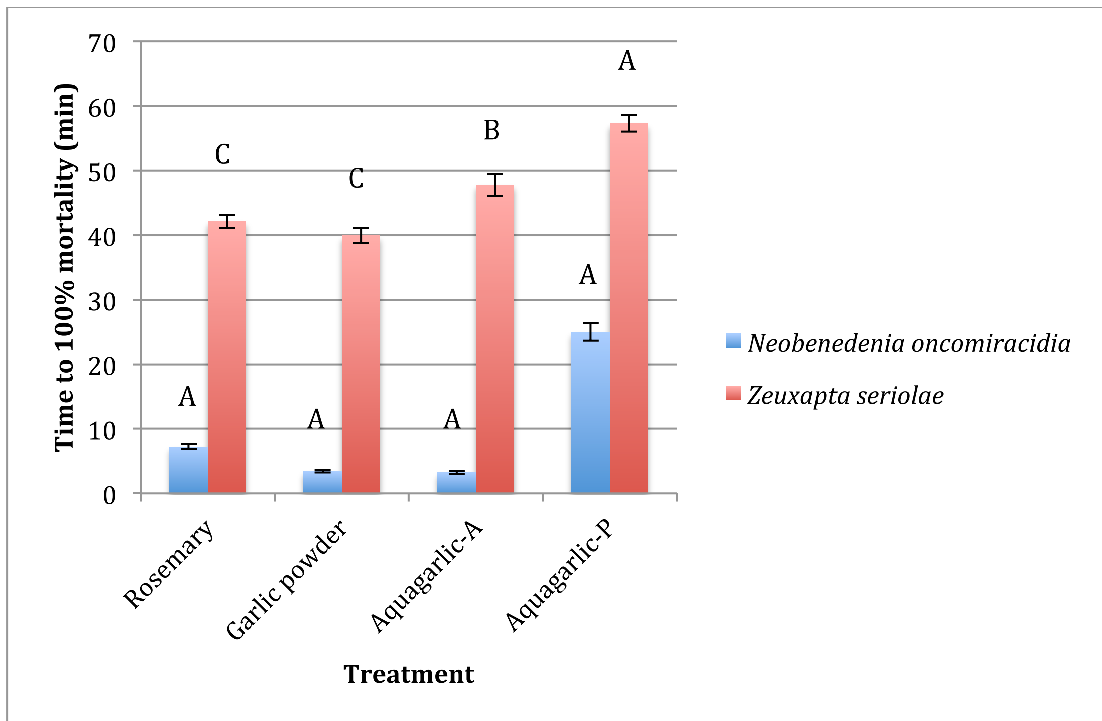

Figure 3.1 Mean (±SE) time to 100% mortality of *Neobenedenia girellae* oncomiracidia and mature *Zeuxapta seriolae* flukes *in vitro*, when exposed to 5 mL seawater solution containing rosemary, garlic powder, Aquagarlic-A, or Aquagarlic-P (n = 3 replicates; 30 parasites per replicate). Controls are not presented due to scale. Bars of the same colour with the same letters not significantly different by Tukey's HSD test.

3.2 *In vivo* trials incorporating anthelmintic herbal extracts into diet

3.2.1 *Trial 1 – herbal extracts as prophylactic treatment for* Zeuxapta seriolae *infections*

The weights of yellowtail kingfish one day prior to the commencement of the trial did not significantly differ between treatment diets (mean = 148.8 ± 1.8 g; $F_{3,12}$ = 1.8, p = 0.19). There was a significant difference in absolute weight gains (g) of yellowtail kingfish fed the different diets over the 30-day conditioning period ($F_{3,12}$ = 5.0, p = 0.02), with the weight gain of fish fed the rosemary extract supplemented diet being significantly less than fish fed the Aquagarlic-P supplemented diet (Figure 3.2; Table 3.2).

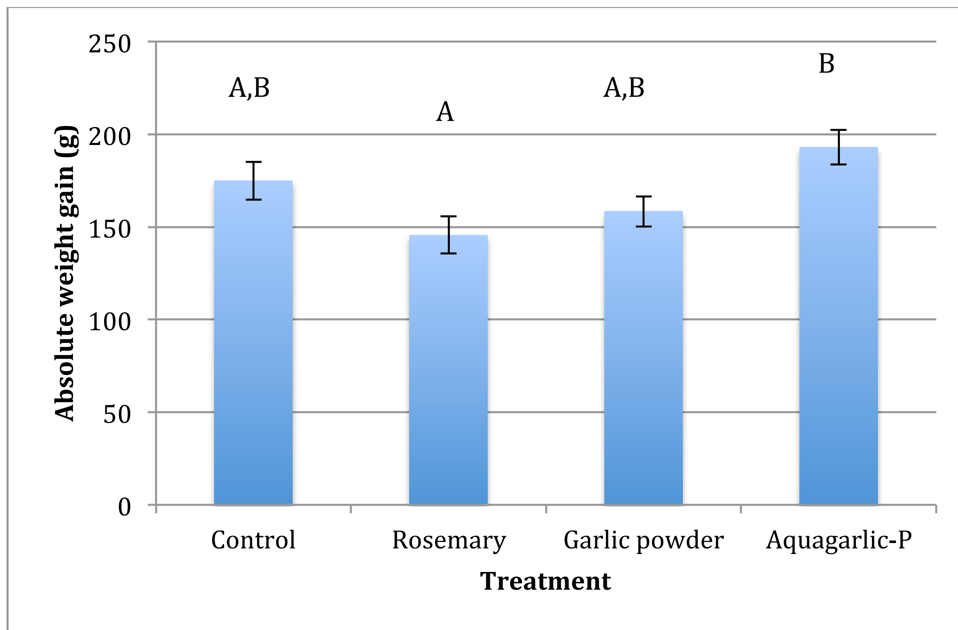

Figure 3.2 Mean (±SE) weight gain (g) of yellowtail kingfish after 30 days of feeding on various herbal supplemented diets (n = 4 replicates, 8 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test.

The feed conversion ratio (i.e. average weight of feed consumed per 1.00g weight increase) ranged from 1.3 in fish fed Aquagarlic-P supplemented feed, to 1.6 in fish fed the diet supplemented with rosemary extract (Table 3.2), with the only significant difference occurring between these two treatments (rosemary and Aquagarlic-P ($F_{3,123}$ = 2.7 , p = 0.04).

Table 3.2 Average feed consumed by yellowtail kingfish to result in1.0g increase in body weight and absolute weight gain (g) of yellowtail kingfish (n = 4 replicates, 8 fish per replicate). *The average water temperature over the 30-day conditioning period was noted at 21.3^{0} C

Diet	Feed conversion ratio	Absolute weight gain (g)
Control	1.4	174.5 ± 10.3
Rosemary extract	1.6	145.6 ± 9.0

Garlic powder	1.5	158.4 ± 8.5
Aquagarlic-P	1.3	192.9 ± 9.3

Following parasitic challenge, all fish fed on the control conditioning diet were infected with *Z. seriolae*, while infection prevalence in fish fed herb-supplemented diets ranged from 72.4 - 96.9% (Table 3.3). There was a significant effect of diet on *Z. seriolae* abundance ($\chi^2_3 = 389.0$, $p < 0.0001$). Parasite abundance (flukes per fish) was significantly less in fish fed all herbal treatment diets compared to the control diet, and on fish fed the diet supplemented with rosemary extract, compared to the diets supplemented with garlic powder Aquagarlic-P (Table 3.3, Figure 3.3).

Table 3.3 Prevalence and abundance of *Zeuxapta seriolae* infecting yellowtail kingfish fed either a control diet or herbal supplemented diet for 30 days prior to challenge (n = 4 replicates; 8 fish per replicate).

Diet	Prevalence (%)	Abundance
Control	100.0	18.6 ± 2.2
Rosemary	72.4	3.5 ± 0.7
Garlic Powder	85.2	5.0 ± 0.9
Aquagarlic-P	96.9	6.5 ± 1.1

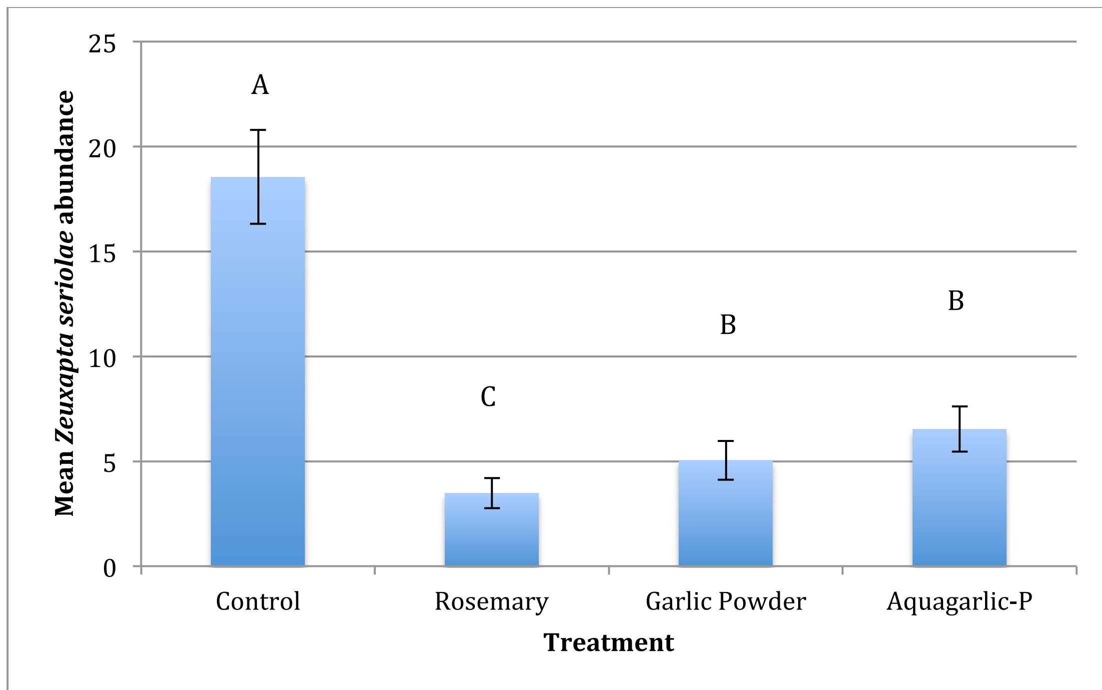

Figure 3.3 Mean (±SE) *Zeuxapta seriolae* abundance on yellowtail kingfish fed either the control or one of three herbs supplemented diets over a 30-day conditioning period before parasite challenge (n = 4 replicates; 8 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test.

3.2.2 Trial 2 – herbal extracts as potential curative treatment for Zeuxapta seriolae *infections*

Prior to commencing the curative trial, the mean abundance of *Z. seriolae* was 170.5 ± 48.1 juvenile parasites per fish. After 10 days feeding, there was no significant effect of treatment diet on parasite abundance (Table 3.4; $\chi^2_3 = 3.3$, $p = 0.34$), but after 20 days of feeding, parasite abundance was significantly different between diets (Table 3.4; $\chi^2_3 = 91.8$, $p < 0.0001$). Parasite abundance was significantly reduced in fish fed all herbal treatment diets, compared to the control diet, but there was no difference in parasite abundance among fish fed the different herbal treatments (Figure 3.4).

Table 3.4 Abundance of mature *Zeuxapta seriolae* infecting yellowtail kingfish fed either a control diet or herbal supplemented curative treatment diet for 10 days and 20 days (n = 3 replicates; 6 fish per replicate).

Diet	Abundance after 10 days	Abundance after 20 days
Control	100.5 ± 11.7	100.5 ± 7.0
Rosemary extract	97.0 ± 12.6	76.3 ± 6.7
Rosemary oil	103.9 ± 13.4	83.8 ± 7.6
Garlic powder	100.5 ± 10.0	74.2 ± 6.3

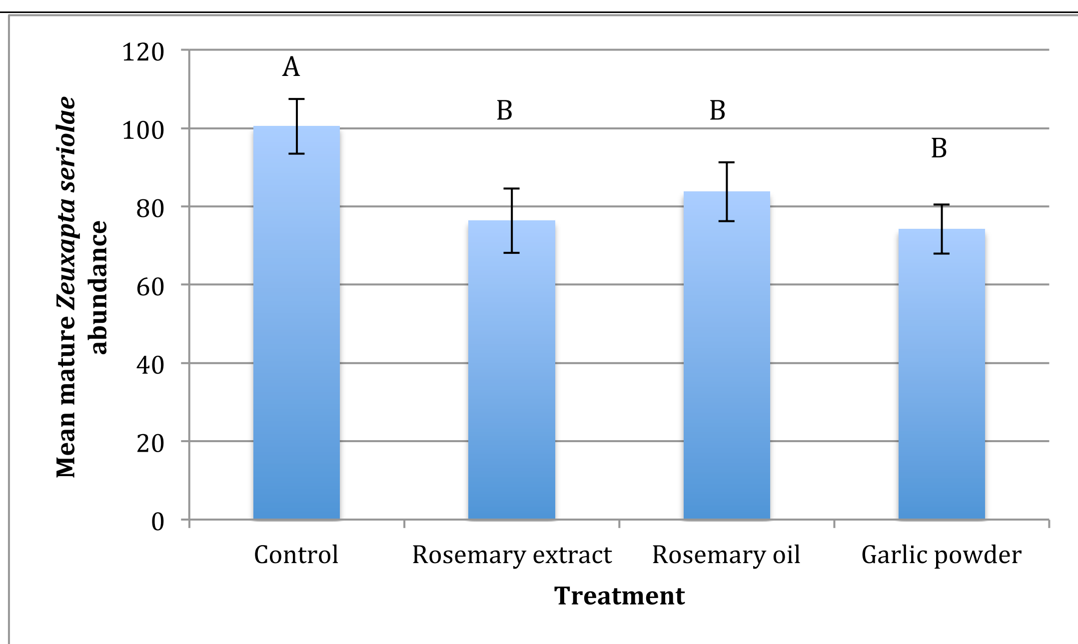

Figure 3.4 Mean (±SE) mature *Zeuxapta seriolae* abundance on yellowtail kingfish fed either the control or one of three herb supplemented curative treatment diets after 20 days of feeding (n = 3 replicates; 6 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test

I noted the presence of only a single generation of gill flukes on yellowtail kingfish at both one-day prior to the commencement of the trial, and at 10 days. However, due to the spawning of mature flukes during the trial, there was a presence of two generations of flukes after 20 days. I differentiated counts between the first and second-generation flukes and separately analyzed the

abundance of second-generation flukes in order to also determine the effects of treatment diets on juvenile *Zeuxapta* recruitment.

The prevalence of juvenile *Z. seriolae* was 100% across all treatments except for rosemary extract (94%). There was a significant effect of treatment on abundance of juvenile *Z. seriolae* after 20 days of feeding ($\chi^2_3 = 1103.0$, $p < 0.0001$), with the lowest recruitment on fish fed rosemary extract and the highest on fish fed the control diet (Table 3.5). There was no significant difference in recruitment of juvenile *Z. seriolae* between fish fed the control diet or a diet supplemented with rosemary oil, but recruitment was significantly less on diets supplemented with rosemary extract or garlic powder (Figure 3.5).

Table 3.5 Abundance of juvenile *Zeuxapta seriolae* infecting yellowtail kingfish fed either a control diet or herbal supplemented curative treatment diet for 20 days (n = 3 replicates; 6 fish per replicate).

Diet	Abundance
Control	129.6 ± 23.4
Rosemary extract	47.1 ± 12.5
Rosemary oil	121.2 ± 26.5
Garlic powder	63.1 ± 13.8

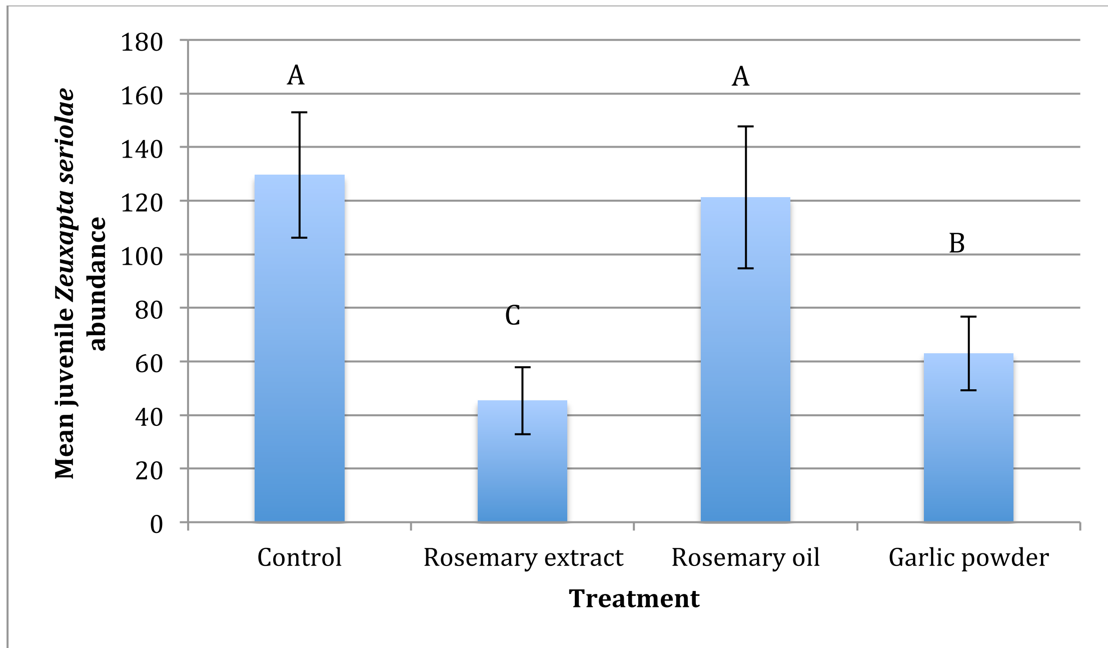

Figure 3.5 Mean (±SE) juvenile *Zeuxapta seriolae* abundance on yellowtail kingfish either the control or one of three herb supplemented curative treatment diets after 20 days of feeding. Bars with the same letter not significantly different by Tukey's HSD test.

4 Discussion

Integrated Pest Management (IPM) involves utilizing all possible techniques to keep pest populations below a level causing economic injury (Dent, 1995). While our extracts did not show 100% success as prophylactic or curative treatments, the efficacy of these extracts lend scientific support for the use of medicinal herbs in controlling the impact of monogenean infections on cultured fish to below a level causing severe economic harm.

4.1 Anthelmintic assessment of herbal extracts *in vitro*

The *in vitro* trial provided baseline data indicating whether the presence of these extracts inhibit survival. Exposure of *N. girellae* oncomiracidia, mature *N. girellae* and mature *Z. seriolae* to various herbal solutions *in vitro* resulted in a significant decrease in survival times of parasites in relation to the seawater controls. From the *in vitro* trial, there was no conclusive evidence of any difference between rosemary extract and the various garlic products in their anthelmintic activity with respect to *Neobenedenia girellae* and *Zeuxapta seriolae.*

Observations on the effectiveness of rosemary and garlic solutions in reducing the survival of *N. girellae* and *Z. seriolae in vitro* are consistent with previous findings on the anthelmintic effects of rosemary and garlic on other species of monogenean parasites (Schelkle et al., 2013; Militz et al., 2013b; Zoral et al., 2017). Previous research into the efficacy of garlic products on the survival time of the monogenean *Gyrodactylus turnbulli in vitro* placed survival of parasites at less than one hour after exposure to solutions containing minced garlic (0.7mg/ml) and garlic granules (0.7mg/ml), compared to a survival of >13 hours for the control (Schelkle et al., 2013). My study found that parasite exposure to garlic products (garlic powder 10 g/L Aquagarlic-A 3 g/L, Aquagarlic-P 3 g/L), as part of a seawater solution, significantly lowered the survival of *N. girellae* oncomiracidia (from 10 hours to <30 min) and mature *Z. seriolae* flukes (from 36 hours to <1h) *in vitro*.

Zoral et al. (2017) examined the anthelmintic effects of rosemary/rosemary extracts on the monogenean *Dactylogyrus minutus* and found that parasite survival to be lower than 10 minutes after exposure to rosemary extract

(200g/L). The present study builds on these observations, showing that survival time after exposure to a rosemary-seawater solution (200g/L) is less than one hour for two species of mature monogeneans: *Neobenedenia girellae* and *Z. seriolae*, and less than 10 minutes for *N. girellae* oncomiracidia. However, while effective at reducing parasite survival *in vitro*, survival of *D. minutus* was shown to be lower than both *N. girellae* and *Z. seriolae*. While I cannot statistically compare the survivals of the monogeneans used in my *in vitro* trial against the survival of *D. minutus* in Zoral et al., (2017) due to lack of data for the latter, it seems to be a sizeable difference in survival times i.e. 7.8 ± 1.4 min (*D. minutus*) compared to 53.3 ± 1.6 min (*N. girellae*) and 42.1 ± 1.0 min (*Z. seriolae*). This difference may be related to the lower survival of *D. minutus* used by Zoral et al., (2017) *in vitro* (control >1200 min) compared to *N. girellae* and *Z. seriolae* (control > 2160), although more research is required to confirm this. *N. girellae* oncomiracidia were more susceptible to herbal solutions than their mature counterpart, a result similar to previous observations that showed *N. girellae* oncomiracidia to be highly susceptible to garlic extract with an allicin concentration of 15.2 µL L^{-1} (garlic = 0.2g/ml)(survival < 2h)(Militz et al., 2013b).

Previous studies have highlighted the pure compounds of rosemary and garlic that are the most effective at reducing monogenean survival *in vitro* (1,8-Cineole and allicin respectively) compared to the other pure compounds that make up the herbs (Schelkle et al., 2013; Militz et al., 2013b; Zoral et al., 2017). Future work should determine the efficacy of rosemary and garlic products on mature *N. girellae* (garlic products only) and juvenile *Z. seriolae* flukes.

4.2 *In vivo* trials incorporating anthelmintic herbal extracts into diet

4.2.1 *Trial 1 – herbal extracts as prophylactic treatment for* Zeuxapta seriolae *infections*

Yellowtail kingfish fed a diet supplemented with rosemary extract gained significantly less weight over a 30-day conditioning period than fish fed a diet containing the commercially manufactured garlic product Aquagarlic-P. Fish fed a diet supplemented with rosemary extract also consumed less feed over the conditioning period than fish on the Aquagarlic-P diet and had a significantly higher (i.e. worse) feed-conversion ratio. There was no difference in weight gain or feed conversion ratio, however, between fish fed the diet supplemented with rosemary extract and those fed the control diet.
This study is the first to show that rosemary extract supplementation (168.5ml/kg) into the diet of yellowtail kingfish does not result in a decrease in fish growth compared to a normal diet, after a 30-day conditioning period.

Yellowtail kingfish fed diets supplemented with garlic products (garlic powder = 10g/kg and Aquagarlic-P = 3g/kg) did not gain significantly more weight than control yellowtail kingfish over the 30-day conditioning period, nor was there a significant difference in the feed-conversion ratios. This contrasts with previous research by Shalaby et al. (2006), who showed that tilapia had a significantly higher growth performance when fed a fixed ration (3% body weight) of a diet supplemented with garlic, with the amount of garlic varying from 10g/kg to 40g/kg, for 90 days. The conflicting results in growth performance may be the

result of differing *in vivo* trial period lengths: 30 days in my trial compared to 90 days in the trial by Shalaby et al. (2006).

Future research into the impacts of herbs on growth performance may consider trialing garlic and rosemary supplementation over a longer period, in order to better determine the effects these diets will have over the course of rearing larvae to table-size fish. Subsequent experiments should also incorporate feeding fish to satiety, in order to accurately determine the impact of rosemary on fish appetite, as garlic has already been shown to stimulate appetite (Lee & Gao, 2012).

This study is the first to show that rosemary, when supplemented into commercial fish feed, reduces infection of *Z. seriolae* on yellowtail kingfish when fed prior to fluke exposure. Supplementation of rosemary extract (168.5 mL/kg) into Ridleys™ pellets significantly reduced parasite abundance by almost six times (from 18.55 to 3.48 flukes per fish), and reduced the prevalence of *Z. seriolae* from 100% to 72.41%. Supplementation of the products containing allicin (garlic powder and Aquagarlic-P) into yellowtail kingfish feed also significantly reduced gill fluke abundance to 5.04 and 6.53 respectively. The results of this study re-enforce the results of Militz et al. (2013b), who showed that supplementation of garlic (50 ml/kg and 150 ml/kg) into the diet of farmed barramundi (*Lates calcarifer*) for 30 days significantly reduced *Neobenedenia* infection success. The results of this study indicate that rosemary and garlic can be successfully utilized in the IPM of monogeneans, and may prove most effective in preventing parasite infections when combined with each other, or

other potential prophylactics, although future research is required to examine the duel use of rosemary and garlic combined.

4.2.2 *Trial 2 – herbal extracts as potential curative treatment for* Zeuxapta seriolae *infections*

The curative effects of rosemary and garlic on existing *Z. seriolae* infections incorporated a method of coating the various herbal products onto the exterior of Ridleys™ pellets using 15 mL/kg of fish oil in order to investigate a practical method that industry could adopt to apply the treatment, as opposed to the lengthy procedure of grinding and reconstructing pellets with the various herbal supplements. Rosemary extract (168.5 mL/kg), rosemary oil (0.1 mL/kg), and garlic powder (10 g/kg) significantly reduced *Z. seriolae* parasite burdens on yellowtail kingfish, when fed to fish for 20 days. These results support the suggestions that rosemary and garlic are beneficial to fish health, by providing protection against pathogens (Nya et al., 2010; Militz et al., 2013b; Zoral et al., 2017). Previous research by Zoral et al. (2017) has shown supplementation of rosemary extract (1,8-Cineole: 31mg/kg) into fish feed for 30 days significantly reduced burdens from the monogenean *Dactylogyrus minutus* on common carp (*Cyprinus carpio*). The present study builds on this, establishing the necessary number of days treatment diets must be fed to fish in order for the therapeutic effects to take hold on yellowtail kingfish monogenean infections (≤20 days).

The curative treatment effects of garlic against a number of different pathogens have been well described for a variety of fish (Nya & Austin, 2009; Militz et al.,

2013b), with garlic doses as low as 5 g/kg enough to control infections of the bacteria *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss*) after a 14-day treatment period (Nya & Austin, 2009). My data highlight the efficacy of garlic on gill flukes *in vivo*, after a 20-day treatment period, and subsequently increases the pool of species to which a garlic treatment can be successfully applied.

This study also found that neither rosemary extract nor garlic powder reduced gill fluke burdens on yellowtail kingfish after a 10-day conditioning period. This corroborates work by Militz et al. (2013b), who showed that a 10-day conditioning period is not long enough for garlic extracts (≤150 mL/kg) to prevent *N. girellae* infections on barramundi.

My study is the first to show that rosemary extract and garlic powder significantly reduced recruitment success of juvenile *Z. seriolae* infecting yellowtail kingfish, after a 20-day feeding period. Recruitment of juvenile *Z. seriolae* was significantly reduced compared to controls after the 20-day feeding trial, supporting my findings from trial 1 and indicating that dietary supplementation of both rosemary extract (168.5 mL/kg) and garlic powder (10 g/kg) onto yellowtail kingfish pellets via fish oil coating may reduce infection in farmed systems.
The compound 1,8-Cineole has been shown to directly inhibit monogenean survival *in vitro* (Zoral et al., 2017), and 31 mg/kg 1,8-Cineole has been shown to significantly lower monogenean infection success and treat pre-existing monogenean infections *in vivo* (see this study; Zoral et al., 2017). It is likely that

the same mechanisms of rosemary extract responsible for their prophylactic effects on gill flukes (as described in this study) are also responsible for the low re-infection of juvenile *Z. seriolae* observed here, although further research is required to confirm this.

Past research has already demonstrated the effects of garlic products on monogenean infection success (Militz et al., 2013b), and the active component allicin has been shown to directly inhibit monogenean survival *in vitro* (Schelkle et al., 2013). My results help confirm that garlic products containing allicin negatively affect monogenean infection success.

In contrast to the results with rosemary extract and garlic powder, supplementation of rosemary oil (0.1 mL/kg) did not inhibit juvenile *Z. seriolae* recruitment after a 20-day feeding period. This low anthelmintic activity may be related to the volume of the treatment supplemented onto yellowtail kingfish pellets, with 0.1 mL/kg too low a ratio to uniformly disperse the treatment throughout the feed using a laboratory mixer. However, the results show that rosemary oil (0.1 mL/kg) can be used to treat infections from adult *Z. seriolae*, so future research is required in order to determine the reason behind the low efficacy of rosemary oil on juvenile *Z. seriolae*. One explanation for the low efficacy surrounding the rosemary oil treatment on juvenile *Z. seriolae* is a lower concentration of 1,8-Cineole than the manufacturer claims. I was unable to quantify the level of 1,8-Cineole and allicin in the rosemary and garlic products respectively, and my methods were based on the assumptions that the concentrations given by the manufacturer were accurate and that replicating the

methods of rosemary extract construction would result in a similar 1,8-Cineole concentration, as determined by previous studies (Zoral et al., 2017). Future research examining the concentrations of 1,8-Cineole and allicin in the herbal products used in this study should clarify this. Future research should also examine the effects of herb-supplemented diets on hatch rates and other indirect causes of low juvenile recruitment, in order to determine the cause of our observations. Again, my results indicate the potential use of rosemary and garlic in IPM of monogeneans in aquaculture. While effective at reducing the abundance of *Z. seriolae* infections, the curative treatments may prove to be more effective when used in tandem with each other or other curative treatments; however, more work is required to confirm this.

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Appendix 7 - Ingelbrecht et al 2020

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Anthelmintic herbal extracts as potential prophylactics or treatments for monogenean infections in cultured yellowtail kingfish (*Seriola lalandi*)

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1. Introduction

Yellowtail kingfish, *Seriola lalandi*, is a pelagic, piscivorous, marine finfish, distributed globally in subtropical and temperate latitudes (Bowyer et al., 2012; Moran et al., 2007). The species is valuable for commercial aquaculture due to its high growth rate and adaptability to sea-cage conditions (Fowler et al., 2003; Poortenaar et al., 2001; Stuart and Drawbridge, 2013). In Australia, the majority of yellowtail kingfish aquaculture currently occurs in the cool-temperate waters of South Australia, where it is the second largest (and most rapidly growing) aquaculture sector (Tanner and Fernandes, 2010), with an annual production of over 4000 tons (Savage and Hobsbawn, 2015). The industry is also expanding into New South Wales and Western Australia (Booth et al., 2010), where warmer waters favor an increase in feeding activity and growth rate.

In the yellowtail kingfish industry, parasites are the most important disease-causing agents affecting production (Ernst et al., 2002). Juvenile yellowtail kingfish for industry are produced in land-based hatcheries, and generally isolated from parasite infections through biosecurity measures (Hutson et al., 2007a). Once fingerlings are moved to sea cages, however, they are susceptible to the parasites present in the local environment (Hutson et al., 2007a). Wild fish passing within close proximity to sea-cages are believed to be the primary source of infection in sea-cage aquaculture (Hutson et al., 2007a; McVicar, 1997).

Forty-two parasite taxa have been reported to infect wild yellowtail kingfish throughout their biogeographic distribution (Ernst et al., 2002; Sharp et al., 2004). Of these, three species of monogeneans (*Benedenia seriolae, Neobenedenia girellae* and *Zeuxapta seriolae*) are the most significant in cultured yellowtail kingfish and require considerable management (Hutson et al., 2007b; Sharp et al., 2003). Treatments for monogenean infections can be classified as prophylactic or curative. Prophylactic treatments are typically administered orally whereas curative treatments may be administered orally or *via* bathing. Orally administered treatments, whether they be prophylactic or curative, are preferred over bath treatments, as no additional infrastructure or handling of livestock is required. In-feed treatment also takes less time to administer and is delivered directly to the host (rather than to the host's environment). Prophylactic treatments are preferable to curative treatments because they avoid the stresses caused by parasite burdens (Dunn et al., 1990; Militz et al., 2013); however, no prophylactic treatments to monogeneans have been identified in yellowtail kingfish aquaculture. This is somewhat due to difficulties associated with practical applications of current synthetic anthelmintics, such as palatability, and treatment costs (Militz et al., 2013; Partridge et al., 2014; Williams, 2010; Williams et al., 2007). In addition, it is necessary to consider the food safety issues caused by the residues of chemotherapeutic agents. These and other undesirable side effects of chemotherapeutic treatments have prompted efforts to explore alternative prophylactics to combat monogenean infections in aquaculture (Lee and Gao, 2012).

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Herbs and herbal extracts have been used in a variety of human and veterinary medicines, and are easily accessible throughout the world. Medicinal herbs contain known anti-parasitic and immunostimulant properties (Reverter et al., 2014; Zoral et al., 2017). The compounds derived from herbs are often less expensive to produce than synthetic chemotherapeutics, have been reported to leave far lower toxic residues in fish flesh, and have fewer adverse effects on water quality (Murthy and Kiran, 2013). Furthermore, some medicinal plants have been reported to stimulate appetite, and subsequently encourage weight gain in fish (Reverter et al., 2014).

Rosemary (*Rosmarinus officinalis*) has been shown to possess anti-parasitic, anti-inflammatory, antibacterial, and anticancer properties (Petiwala and Johnson, 2015; Zimmermann, 1980; Zoral et al., 2017). In aquaculture, extracts prepared from rosemary leaves have been used to successfully treat infections from the monogenean *Dactylogyrus minutus* in carp, *Cyprinus carpio* (Zoral et al., 2017); however, there have been no published studies on the use of rosemary to treat monogenean infections in yellowtail kingfish. Of the major components in rosemary extracts, 1,8-Cineole (cineole) has been shown to have the strongest effect on monogeneans (Zoral et al., 2017). Garlic (*Allium sativum*) has also been shown to possess antimicrobial, immunostimulant, and anti-parasitic properties (Lee and Gao, 2012), which are attributed to the phytochemical allicin (diallyl thiosulfinate) and its derivatives (Militz et al., 2013; Nya et al., 2010). Garlic extract has been successfully applied as both a prophylactic and curative treatment for *N. girellae* in barramundi (*Lates calcarifer*) (Militz et al., 2013, 2014).

The aims of this study were to investigate whether oral administration of rosemary and garlic extracts could be viable prophylactic and/or curative treatments against monogenean infections in cultured yellowtail kingfish. We report here the results of three trials. The first trial tested whether such extracts would inhibit the survival of *N. girellae* oncomiracidia and adult *N. girellae* and *Z. seriolae in vitro*. A second trial tested the prophylactic effects of these extracts in preventing monogenean attachment by feeding supplemented diets for 30 days prior to challenge with of *Z. seriolae* oncomiracidia and a third trial investigated whether dietary supplementation with these herbs is effective in treating fish already infected with this parasite.

2. Materials and methods

All *in vivo* trials were conducted under Murdoch University Animal Ethics Approval: R3038/18.

2.1. Anthelmintic assessment of herbal extracts in vitro

Two herbal extracts and two commercially available herbal products were selected for an initial *in vitro* assessment of their anthelmintic properties against *N. girellae* (both mature parasites and oncomiracidia) and *Z. seriolae* (mature parasites only). The products tested were dry rosemary leaves (Spencers™, Anchor Foods, Fremantle, Australia), dried ground garlic (Spencers™, Anchor Foods, Fremantle, Australia) and two commercial products containing organosulphur products derived from allicin (Aquagarlic-P and Aquagarlic-A, Domca S.A.U., Granada, Spain). Solutions of each product were prepared by stirring the specified quantity (based on previous research or manufacturer recommendations; Table 1) with 100 mL of seawater at 100 °C for 10 min. The supernatants were then filtered through 2 μm syringe filters, and stored at 4°C until use.

Parasites were exposed to each treatment (mature *N. girellae* were exposed only to rosemary solution due to insufficient numbers of this parasite lifestage) and to seawater controls in triplicate in 6-well cell culture plates, with 30 individual parasites per well. Newly hatched *N. girellae* oncomiracidia were isolated *via* pipette from eggs incubated in culture plates of seawater. Adult *N. girellae* and *Z. seriolae* of the same apparent size and maturity classes were collected from the skin and gills, respectively, of naturally infected yellowtail kingfish. Parasites were stocked into wells in 1 mL of seawater, then 5 mL of the allocated treatment solution (or seawater) was added. Parasites were monitored under a dissecting microscope; constantly during the first hour, then hourly for 36 h. Death of the parasites was judged by cessation of movement. The time was recorded when 100% of the parasites in each well were dead. The time of the first parasite mortality in each well was also recorded to determine the range of times to death.

Table 1
Dosage of herbal ingredient stirred into 100 mL seawater for 10 min at 100°C for *in vitro* trials.

Herbal ingredient	Dose g/100 mL seawater
Dry rosemary leaves	20.0
Garlic powder	1.0
Aquagarlic-A	0.3
Aquagarlic-P	0.3

2.2. In vivo Trial 1 – herbal extracts as prophylactic treatments against Zeuxapta seriolae infections

The first *in vivo* trial investigated the potential of herbal products as prophylactics against infection success of *Z. seriolae* oncomiracidia. The trial compared three treatment diets against an unmedicated control diet. Treatment diets included rosemary, garlic or Aquagarlic-P. Both treatment and control diets were prepared by firstly grinding commercial yellowtail kingfish feed (Pelagica, Ridley Agriproducts Naragnba Queensland,) in a mill. Dried ground garlic and Aquagarlic-P were added directly to the ground feed at 10 g/kg (Aly et al., 2008) and 3 g/kg (manufacturer's recommendation), respectively. Water was added to the dry mash at 450 mL/kg and mixed for 10 min to form a dough before forming into pellets using a pasta extruder (Dolly LaMonerrina, Lineapasta S.R.L, Citadella, Italy). For the rosemary diets, dried rosemary leaves were extracted into ethanol, then added to the feed as a liquid following Zoral et al. (2017). This herbal extract was added to the milled feed at 168 mL/kg along with 282 mL/kg of water (to achieve the same total volume of 450 mL/kg) before pelletising as described above. This quantity of rosemary extract was selected to match the successful dietary inclusion level of cineole identified by Zoral et al. (2017) of 3.1 mg per 100 g feed, and on the assumption that our rosemary-ethanol extract would have a similar cineole content of 0.18 mg/mL. Ethanol extraction was used, rather than seawater as in the *in vitro* trial, because it is a more efficient way of extracting cineole from rosemary leaves (Zoral et al., 2017). To incorporate a successful cineole concentration into our diet using water extraction, we would have had to exceed the optimal addition of 450 mL/kg of water to the dry mash, which would have compromised the state of the diet before pellet extruding. The un-medicated, control diet was prepared using the same methodology, without the addition of a herbal supplement and with 450 mL/kg of water. After extruding mixtures into pellets, all pellets were air-dried over 3 days, after which all diets exhibited moisture contents < 10%.

Juvenile yellowtail kingfish (mean weight 148 g), free of parasite infections as a result of being reared in a land-based hatchery using filtered bore-water, were obtained from the Australian Centre for Applied Aquaculture Research in Fremantle, Western Australia and randomly distributed among twenty 300 L tanks (8 fish per tank). All fish were feeding normally, and detailed histopathological health checks on a sample of the same cohort of fish found no health problems. During a 3-day acclimation period to the experimental conditions, the fish were fed to satiety once daily on unmedicated commercial yellowtail kingfish feed (Ridley Agriproducts, Pelagica 3 mm). One day prior to commencing the trial, fish were anaesthetized (20 mg/L AQUI-S, Lower Hutt, New Zealand) and individually weighed. During the 30-day trial, fish were fed a fixed ration of the treatment diets. The ration

began at 50 g/tank/day (4.2% BW/day) and increased by 5 g/tank each week. Fish were fed half of the fixed ration at 0900 and 1500 h. Any pellets that were not consumed were removed 5 min after feed was first administered, with the amount of uneaten pellets recorded. Tanks operated on flow through, with each tank receiving 180 L/h of new, filtered seawater. Dissolved oxygen was maintained at > 80% oxygen saturation at all times and during the trial water temperature averaged 21.9 °C.

At the end of the trial, fish were again anaesthetized and individually weighed before being returned to their respective tanks. The incoming water to each tank was stopped and 400 *Z. seriolae* oncomiracidia (obtained from eggs incubated in culture plates of seawater) were added to each tank. Prior to addition, oncomiracidia were individually isolated and counted by pipette under a light microscope.

Water flow remained off for 2 h in order to give the oncomiracidia time to seek out and attach to a host. Fish were fed to satiety with commercial (unmedicated) feed for a further 14 days to allow attached parasites to grow to a size at which they could be easily counted, but before they could reproduce within the experimental tanks. After this period, each fish was euthanized *via* an anesthetic overdose (40 mg/L AQUI-S) then placed into a bath containing dissolved praziquantel (50 mg/L) for 10 min to remove any attached parasites (Hutson, 2007; Mansell et al., 2005). These parasites from each individual fish were sieved out and fixed in 70% ethanol for subsequent counting.

2.3. In vivo Trial 2 – herbal extracts as therapeutic treatments against Zeuxapta seriolae infections

The second *in vivo* trial investigated the potential of herbal products as therapeutics against existing infections with *Z. seriolae*. Three treatment diets and one unmedicated control diet were included. Based on the results of *in vivo* Trial 1 and in order to investigate a more commercially relevant form of rosemary, Aquagarlic-P was replaced with a commercial rosemary oil (essential rosemary oil, Range Products, Welshpool, Australia). The same ground garlic and ethanolic rosemary extract treatments and inclusion levels tested in Trial 1 were included (10 g/kg and 168.5 mL/kg respectively). Treatment diets were prepared by mixing commercial yellowtail kingfish diets (9 mm Pelagica, Ridley Agriproducts, Narangba, Australia) with 15 mL/kg of fish oil for 10 min with the relevant quantity of the herbal treatment. Control diets were also mixed with 15 mL/kg of fish oil. The amount of rosemary oil added (0.1 mL/kg) was based on the manufacturer's declared cineole content (30%), to achieve the same aforementioned optimum dietary cineole content of 3.1 mg per 100 g feed.

Yellowtail kingfish (mean weight 700 g), naturally infected with *Z. seriolae* were transferred from commercial seacages and randomly allocated to twelve, 4000 L research tanks (12 fish per tank). Ten additional fish from the same seacage were collectively anaesthetized (40 mg/L AQUI-S) and bathed in praziquantel as previously described in order to provide a count of the mean number of parasites per fish at the start of the trial. Seawater (temperature 21.5 °C) flowed through each tank at 1200 L/h and dissolved oxygen was maintained at > 80% saturation at all times. Fish were acclimated to the experimental system for 3 days during which time they were fed to satiety once daily on unmedicated commercial feed. During the 20-day experimental period, fish were fed the treatment diets to satiety twice daily. At day 10 of the trial, 50% of the fish from each tank were randomly selected, euthanized and bathed in praziquantel as previously described. At the end of the 20-day trial, the remaining fish were sampled with the same method. All removed parasites were counted and categorized as either juvenile or mature, with parasites being considered mature at approximately 2 mm and when evidence of egg production has started (Mansell et al., 2005).

2.4. Determination of cineole and allicin quantities in treatments

Following the conclusion of *in vivo* trials, the concentrations of cineole and allicin in rosemary and garlic were determined. The cineole content of rosemary extracts and rosemary oil were analyzed *via* gas chromatography and mass spectrometry (GC–MS) using methods described by Zoral et al. (2017).

Allicin content of the dried ground garlic was analyzed *via* gas chromatography and flame ionization detection (GC-FID). To 1 g of garlic powder, 50 mL of phosphate buffer (0.1 mol/L) (pH 7) was added and incubated for 5 min at 35 °C before being filtered. A 25 mL sample of the filtrate was then mixed with 10 mL sodium borohydride (0.2 mol/L) and 40 mL ethyl acetate, in order to reduce the allicin in the sample to diallyl disulfide. The solution was shaken for 10 min, before being dehydrated using 1 mL internal standard (1% isomyl alcohol). The mixture was then transferred to a measuring flask and diluted to 50 mL with ethyl acetate. The GC analysis determining the amount of diallyl disulfide in the sample was conducted using a flame ionization detector (FID), with GC separations performed on a DB-1 column (0.53 mm × 30 m), using helium as the carrier gas. The column temperature was programed to increase from 50 °C to 320 °C over a 30-min period. The detector interface temperature was 320 °C.

2.5. Statistical analyses

Data for each of the trials were analyzed using JMP v10 (SAS Institute, Carey, NC) and R v6.1.15 (R Core Team, 2015). Differences in survival time of parasites among *in vitro* treatments were compared by the Kaplan–Meier method, with a Hochberg (1988) correction for multiple testing.

Differences in starting weight, absolute weight gain and feed conversion ratio (*i.e.* average weight of feed consumed per 1 g weight increase) among fish fed different preventative treatment diets in the first *in vivo* trial were analyzed with a general linear model, with replicate tanks nested within treatments as a random effect. Residuals from the model were normally distributed. Where a significant effect of treatment was found, Tukey's HSD test was used for post-hoc multiple comparisons.

Differences in abundance of parasites (*i.e.* number of parasites per fish host) among treatment diets in both *in vivo* trials were analyzed using a generalized linear mixed model, with parasite abundance modelled as a negative binomial variable with a log link, and replicate tanks nested within treatments as a random effect. The residual by predicted plot showed a random scatter, with no discernible pattern. Where a significant effect of treatment was found, pairwise contrasts were made among all treatments, using a Bonferroni correction for an experiment-wide Type I error of 0.05.

3. Results

3.1. Anthelmintic assessment of herbal extracts in vitro

Survival time for each of the experimental groups is shown in Table 2. The survival time of mature *N. girellae* was significantly reduced by the rosemary solution compared to the seawater control ($\chi^2_4 = 5.0$, $P = .02$). The survival time of *N. girellae* oncomiracidia differed significantly among treatments ($\chi^2_4 = 32.3$, $P < .0001$), with multiple comparison tests showing that all herbal treatments reduced survival time relative to the control, but there were no significant differences in survival time among the herbal treatments themselves. There was also a significant effect of treatment on the survival time of mature *Z. seriolae* ($\chi^2_4 = 24.9$, $p < .0001$), with all herbal treatments reducing survival time relative to the control, and survival time being the least in the garlic powder and rosemary solutions, followed by Aquagarlic-A and Aquagarlic-P.

Table 2
in vitro effects of various herbal solutions on the survival of *Neobenedenia girellae* oncomiracidia (*Neobenedenia* O), mature *Neobenedenia girellae* (*Neobenedenia* M) (rosemary only), and mature *Zeuxapta seriolae* ($n = 3$ replicates; 30 parasites per replicate).

Treatment	*Parasite* spp.	Time to 100% mortality (min)	Range of survival time (min)
Seawater control	*Neobenedenia* (O)	545.20 ± 27.41	150.38–599.63
	Neobenedenia (M)	> 2160	300 - > 2160
	Zeuxapta	> 2160	780 - > 2160
Rosemary	*Neobenedenia* (O)	7.22 ± 0.40	3.53–7.98
	Neobenedenia (M)	53.25 ± 1.58	18.73–59.09
	Zeuxapta	42.10 ± 1.03	35.33–43.85
Garlic powder	*Neobenedenia* (O)	3.43 ± 0.19	1.98–3.78
	Zeuxapta	39.92 ± 1.15	26.97–41.37
Aquagarlic-A	*Neobenedenia* (O)	3.22 ± 0.23	1.16–3.68
	Zeuxapta	47.77 ± 1.71	38.95–51.05
Aquagarlic-P	*Neobenedenia* (O)	25.03 ± 1.36	15.48–27.30
	Zeuxapta	57.3 ± 1.30	35.23–59.5

3.2. In vivo trials incorporating anthelmintic herbal extracts into diet

3.2.1. Trial 1 – herbal extracts as prophylactic treatments for Zeuxapta seriolae infections

The weights of yellowtail kingfish at the commencement of the trial did not significantly differ between treatment diets (mean = 148.8 ± 1.8 g; $F_{3,12} = 1.8$, $p = .19$). Although there was no significant difference in feed consumption of fish fed the different diets over the 30-day conditioning period, there was a significant difference in the absolute weight gain (g) ($F_{3,12} = 5.0$, $p = .02$), with the weight gain of those fed the rosemary extract supplemented diet being significantly less than those fed the Aquagarlic-P supplemented diet (Fig. 1). Feed conversion ratio ranged from 1.3 ± 0.1 in fish fed Aquagarlic-P supplemented feed, to 1.6 ± 0.1 in fish fed the diet supplemented with rosemary extract, with the only significant difference occurring between these two treatments (Fig. 1; $F_{3,123} = 2.7$, $p = .04$).

Following challenge with *Z. seriolae*, all fish on control diets were infected and the prevalence of infection ranged from 72.2–96.9% in fish fed the herbal treatment diets (Table 3). There was a significant effect of diet on *Z. seriolae* abundance (Table 3; $\chi^2_3 = 389.0$, $p < .0001$). Parasite abundance was significantly less on fish fed all herbal treatment diets compared to the control diet, and on fish fed the diet supplemented with rosemary extract, compared to the diets supplemented with garlic powder or Aquagarlic-P (Fig. 2).

The GC–MS analyses of our rosemary extract and dried ground garlic powder revealed concentrations of 5.02 mg/mL cineole and 3.00 mg/g allicin respectively, which yielded dietary concentrations of 84.59 mg and 3.00 mg, respectively per 100 g of feed.

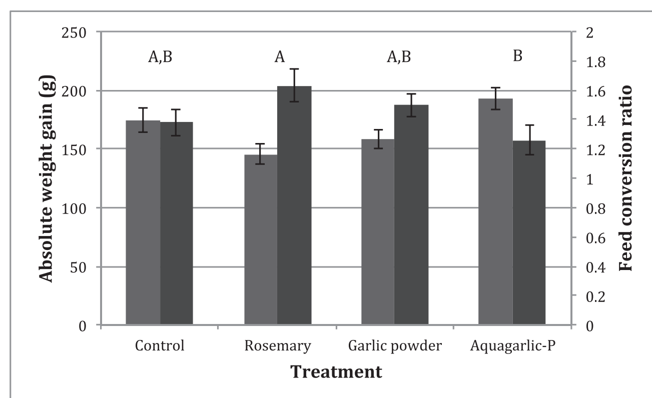

Fig. 1. Mean (± SE) weight gain (g) (left) and feed conversion ratio (right) of *Seriola lalandi* after 30 days of feeding on various herbal supplemented diets ($n = 4$ replicates, 8 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test.

Table 3
Mean abundance and prevalence of *Zeuxapta seriolae* on *Seriola lalandi* fed either the control or one of three herbal supplemented diets for a 30-day conditioning period before parasite challenge ($n = 4$ replicates; 8 fish per replicate).

Diet	Abundance (prevalence %)
Control	18.6 ± 2.2 (100.0)
Rosemary extract	3.48 ± 0.7 (72.4)
Garlic powder	5.0 ± 0.9 (85.2)
Aquagarlic-P	6.5 ± 1.1 (96.9)

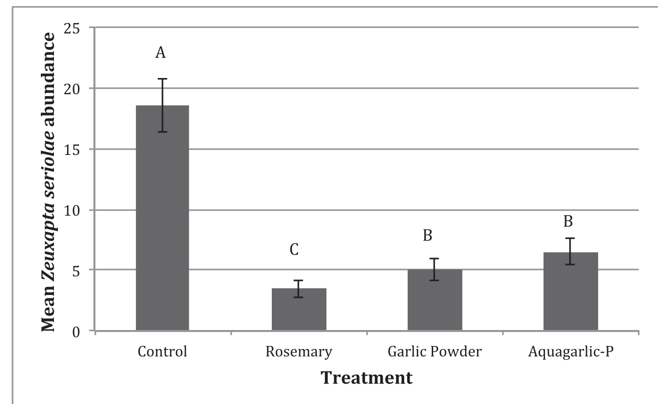

Fig. 2. Mean (± SE) *Zeuxapta seriolae* abundance on *Seriola lalandi* fed either the control or one of three herbal supplemented diets over a 30-day conditioning period before parasite challenge ($n = 4$ replicates; 8 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test.

3.2.2. Trial 2 – herbal extracts as therapeutic treatments for Zeuxapta seriolae infections

Prior to commencing the trial, all fish were infected and the mean abundance (in this case equivalent to intensity) of *Z. seriolae* was 170.5 ± 48.1 parasites per fish. After 10 days feeding, prevalence of infection in fish for all diets was still 100% and there was no significant effect of treatment diet on parasite abundance (Table 4; $\chi^2_3 = 3.3$, $p = .34$), but after 20 days of feeding, parasite abundance was significantly different between diets (Table 4; $\chi^2_3 = 91.8$, $p < .0001$). Parasite abundance was significantly reduced in fish fed all herbal treatment diets, compared to the control diet, but there was no significant difference in parasite abundance among fish fed the different herbal treatments (Fig. 3). Prevalence of infection was 100% in fish for all diets, after 20 days feeding (Table 4).

The presence of only a single generation of gill parasites was observed at both the commencement of the trial, and at 10 days post-treatment. However, due to the reproduction of mature parasites during the trial, two generations of parasites were present after 20 days. The

Table 4
Mean abundance and prevalence of mature *Zeuxapta seriolae* infecting *Seriola lalandi* fed either a control diet or herbal supplemented curative treatment diet for 10 and 20 days ($n = 3$ replicates; 6 fish per replicate).

Diet	Abundance after 10 days (prevalence %)	Abundance after 20 days (prevalence %)
Control	100.5 ± 11.7 (100)	100.5 ± 7.0 (100)
Rosemary extract	97.0 ± 12.6 (100)	76.3 ± 6.7 (100)
Rosemary oil	103.9 ± 13.4 (100)	83.77 ± 7.6 (100)
Garlic powder	100.5 ± 10.0 (100)	74.22 ± 6.3 (100)

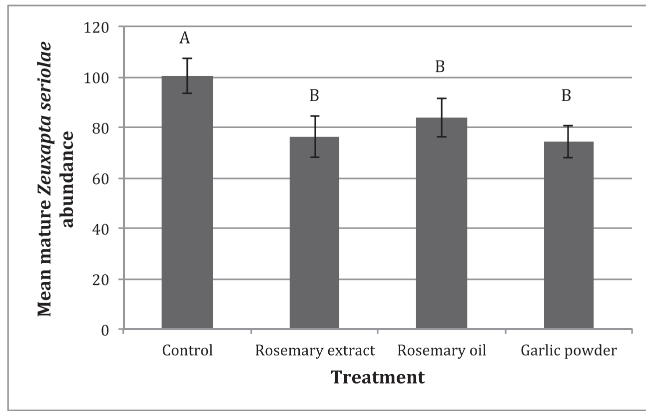

Fig. 3. Mean (± SE) mature *Zeuxapta seriolae* abundance on *Seriola lalandi* fed either the control or one of three herbal supplemented curative treatment diets after 20 days of feeding ($n = 3$ replicates; 6 fish per replicate). Bars with the same letter not significantly different by Tukey's HSD test.

Table 5
Mean abundance and prevalence of juvenile *Zeuxapta seriolae* infecting *Seriola lalandi* fed either a control diet or herbal supplemented curative treatment diet for 20 days ($n = 3$ replicates; 6 fish per replicate).

Diet	Abundance (prevalence %)
Control	129.6 ± 23.4 (100.0)
Rosemary extract	47.1 ± 12.5 (94.4)
Rosemary oil	121.2 ± 26.5 (100.0)
Garlic powder	63.1 ± 13.8 (100.0)

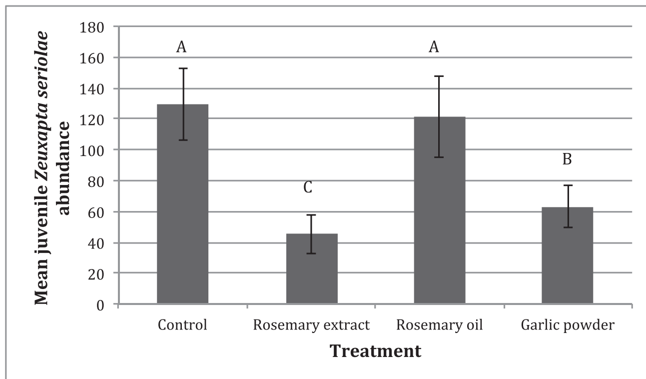

Fig. 4. Mean (± SE) juvenile *Zeuxapta seriolae* abundance on *Seriola lalandi* fed either the control or one of three herbal supplemented curative treatment diets after 20 days of feeding. Bars with the same letter not significantly different by Tukey's HSD test.

first and second-generation parasites were counted separately. There was a significant effect of treatment on abundance of juvenile *Z. seriolae* after 20 days of feeding (Table 5; $\chi^2_3 = 1103.0, p < .0001$). There was no significant difference in recruitment of juvenile *Z. seriolae* between fish fed the control diet or a diet supplemented with rosemary oil, but recruitment was significantly less on diets supplemented with rosemary extract or garlic powder (Fig. 4). Prevalence of juvenile *Z. seriolae* after 20 days ranged from 94.4–100% (Table 5).

Analyses of the amount of cineole and allicin present in our treatments revealed cineole concentrations of 6.06 mg/mL (rosemary extract) and 679 mg/mL (rosemary oil), and an allicin concentration of 3.00 mg/g in garlic powder, which yielded dietary concentrations of 102.11 mg, 6.79 mg and 3.00 mg, respectively per 100 g feed.

4. Discussion

The *in vitro* trial provided baseline data to show that exposure of *N. girellae* oncomiracidia, mature *N. girellae* and mature *Z. seriolae* to various herbal solutions resulted in a significant decrease in survival times of parasites in relation to seawater controls. From the *in vitro* trial, there was no evidence of any difference between rosemary extract and the various garlic products in their anthelmintic activity with respect to *Neobenedenia girellae* and *Zeuxapta seriolae* and therefore all products were considered worthy of investigation *in vivo*.

Observations on the effectiveness of rosemary and garlic solutions in reducing the survival of *N. girellae* and *Z. seriolae in vitro* are consistent with previous findings on the anthelmintic effects of rosemary and garlic on other species of monogenean parasites (Fridman et al., 2014; Militz et al., 2014; Zoral et al., 2017). Previous research has highlighted the benefits of garlic products to combat infectious diseases in aquaculture, with dosages of ≥10 g/kg diet being successful in managing bacterial infections in tilapia (*Oreochromis niloticus*) (Aly et al., 2008). Furthermore, dosages of garlic extract ranging from as low as 7.5 mL/L to 12.5 mL/L have been shown to inhibit survival of the monogenean *Gyrodactylus turnbulli in vitro* (Fridman et al., 2014). The experiments presented here showed that exposure to garlic products (garlic powder 10 g/L Aquagarlic-A 3 g/L, Aquagarlic-P 3 g/L), as part of a seawater solution, significantly lowered the survival of *N. girellae* oncomiracidia (from 10 h to < 30 min) and mature *Z. seriolae* flukes (from 36 h to < 1 h).

Zoral et al. (2017) examined the anthelmintic effects of rosemary extracts on the monogenean *D. minutus in vitro* and found that parasite survival was 7.8 ± 1.4 min after exposure to an aqueous rosemary extract (200 g/L). The present study builds on these observations, showing that survival time after exposure to a similar rosemary-seawater solution (200 g/L) is < 1 h for two species of mature monogeneans: *N. girellae* and *Z. seriolae*, and < 10 min for *N. girellae* oncomiracidia. However, the time to complete mortality of mature *D. minutus* was much faster than the mature *N. girellae* and *Z. seriolae* tested in this study, suggesting either a difference in anthelmintic activity between the aqueous rosemary extract and our rosemary-seawater solution, or that these species are perhaps more tolerant to the herbs than *D. minutus*. Oncomiracidia of *N. girellae* appeared more susceptible to the herbal treatments than mature individuals, a result similar to previous observations that showed *N. girellae* oncomiracidia to be highly susceptible to garlic extract with an allicin concentration of 15.2 μL/L (garlic = 0.2 g/mL; survival < 2 h) (Militz et al., 2014).

Fish fed a diet supplemented with rosemary extract (168.5 mL/kg; 5.02 mg/mL cineole) gained significantly less weight over the 30-day conditioning period than fish fed a diet containing the commercially manufactured garlic product Aquagarlic-P. This can be explained by the significantly poorer feed conversion ratio displayed by fish fed rosemary extract compared to those fed Aquagarlic-P. There was no difference in weight gain or feed conversion ratio, however, between fish fed the diet supplemented with rosemary extract and those fed the control diet. High doses of rosemary aqueous extract (≥20 mL per 100 g feed) have been associated with hepatotoxicity and nephrotoxicity in carp (Zoral et al., 2018). The difference in growth performances between fish supplemented with rosemary extract and Aquagarlic-P may be reflected by the toxicity of rosemary extract; however, further investigation is required to test this hypothesis and establish an effective and safe dosage regimen for yellowtail kingfish.

Fish fed on diets supplemented with garlic products (garlic powder = 10 g/kg and Aquagarlic-*P* = 3 g/kg) did not gain significantly more weight than fish fed a control diet over the 30-day conditioning period, nor was there a significant difference in the feed-conversion ratios. This contrasts with previous studies (Shalaby et al., 2006), which have shown significantly higher growth performance in tilapia when fed a similar fixed ration (3% body weight) of diets supplemented with garlic, with the amounts of garlic varying from 10 g/kg

to 40 g/kg, and diets being fed over 90 days. This may be the result of the shorter trial period here (30 days) relative to 90 days in the trial by Shalaby et al. (2006). It may also simply be that garlic does not have an influence on yellowtail kingfish growth, as previous research has revealed that not all fish growth rates are increased through dietary inclusion of garlic (Thanikachalam et al., 2010). Additional research is required to determine this.

Zoral et al. (2017) demonstrated that cineole was the most effective anthelmintic compound within rosemary and that 3.1 mg of cineole per 100 g of feed is enough to treat the monogenean *D. minutus* in carp. These authors found that ethanol extraction was more effective than aqueous extraction. Despite preparing rosemary extract using the same method described by Zoral et al. (2017), our extract had a cineole concentration over 27 times higher than that found in the rosemary-ethanol extract prepared by these researchers (cineole 0.18 mg/mL). The difference in cineole concentrations in the two extracts may be due to a number of factors; seasonal and geographical variations in harvesting the rosemary leaves, the age of the plant, and slight differences in the storage time between blending and centrifuging the solution can all significantly impact the final concentrations of the pure compounds present in the extract (Lakušić et al., 2013; Perry et al., 1999; Salido et al., 2003). Despite exceeding the intended cineole concentration in the treatment, fish fed with the rosemary extract diet did not display any obvious ill effects (*i.e.* slower growth or mortality) relating to a cineole overdose in relation to fish fed a control diet during the trial. Fish growth for this treatment was, however, significantly less than Aquagarlic-P fish, so we cannot conclusively state that a rosemary extract with our cineole concentrations will have no adverse effects on fish health. An acute toxicity test on yellowtail kingfish using pure cineole would give clarity to any potential impacts this treatment would have on fish production in the long term.

This study showed that rosemary extract with a cineole concentration of 5.02 mg/mL (84.59 mg cineole per 100 g feed), when supplemented into commercial fish feed, reduces infection success of *Z. seriolae* on yellowtail kingfish when fed prior to exposure to infectious oncomiracidia. Supplementation of rosemary extract (168.5 mL/kg) into Ridleys pellets significantly reduced parasite abundance by almost six times (from 18.55 to 3.48 flukes per fish). Furthermore, the results of the *in vitro* trial, showing that rosemary solution in seawater killed the parasite, suggest that the anthelmintic effects of rosemary extract seen *in vivo* are a result of the cineole present in the added extract, rather than trace amounts of ethanol. However, as we used an ethanol extract in the *in vivo* trial (for reasons explained in the Methods), further research is necessary to definitively identify the compound(s) in rosemary responsible for the protective effect against *Z. seriolae*. Supplementation of the products containing allicin (garlic powder and Aquagarlic-P) into feed also significantly reduced gill fluke abundance to 5.04 and 6.53 respectively. The results of this study reenforce the results of Militz et al. (2014), who showed that supplementation of garlic/allicin (50.00 mL/kg and 150.00 mL/kg; 3.40 mg and 10.20 mg allicin per 100 g feed respectively) into the diet of farmed barramundi for 30 days significantly reduced *N. girellae* infection success. We can further report that incorporating allicin at 3.00 mg per 100 g feed can significantly lower *Z. seriolae* infection success on yellowtail kingfish.

Oral administration of rosemary extract (168.5 mL/kg feed; cineole = 6.06 mg/mL), rosemary oil (0.1 mL/kg; cineole = 679.00 mg/mL), and garlic powder (10 g/kg; allicin = 3.00 mg/g) for 20 days also significantly reduced *Z. seriolae* parasite burdens on fish that were already infected. The curative treatment effects of garlic against a number of different monogeneans have been well described for a variety of fish (Fridman et al., 2014; Martins et al., 2002; Militz et al., 2014), with garlic doses as low as 1 g/kg (unspecified allicin concentration) enough to treat *Anacanthorus penilabiatus* infections in Pacu (*Piaractus mesopotamicus*) after 15 days of treatment. The trials presented here support the efficacy of garlic in reducing *Z. seriolae* infection burden *in vivo* after a 20-day treatment period in yellowtail kingfish. None of the herbal supplement treatments reduced *Z. seriolae* burdens after a 10-day conditioning period. This corroborates work by Militz et al. (2014), which showed that a 10-day conditioning period is not long enough for garlic extracts (≤150 mL/kg; ≤10.20 μL allicin per 100 g feed) to prevent *N. girellae* infections on barramundi.

The trials presented here showed that rosemary extract and garlic powder supplemented feed significantly reduced recruitment success of juvenile *Z. seriolae* infecting yellowtail kingfish, after a 20-day feeding period. The compound cineole has been shown to directly inhibit monogenean survival *in vitro* (Zoral et al., 2017), and 3.1 mg of cineole per 100 g of feed has been shown to significantly lower monogenean infection success and treat pre-existing infections *in vivo* (Zoral et al., 2017). It is likely that the same compounds in rosemary extract responsible for the prophylactic effects are also responsible for the low reinfection of juvenile *Z. seriolae* observed here.

In contrast to the results with rosemary extract and garlic powder, supplementation of rosemary oil did not inhibit juvenile *Z. seriolae* recruitment after a 20-day feeding period. This low anthelmintic activity may be explained through the post-trial analysis, which revealed over 15 times less cineole present per 100 g of feed of rosemary oil diet (6.79 mg) compared to the rosemary extract diet (102.1 mg). However, the quantity of cineole in the rosemary oil diet was over double the effective concentration used by Zoral et al. (2017) to treat *D. minutus* in carp, and the rosemary oil diet was successful at reducing the abundance of mature *Z. seriolae*. It could be that juvenile *Z. seriolae* are not only more resistant to cineole than *D. minutus*, but also more resistant than mature *Z. seriolae*. Another explanation may be that there are other pure compounds of rosemary (*i.e.* -β-Pinene) present in the oil that are more potent to mature *Z. seriolae* than juveniles; however, further investigation is required to confirm this.

Integrated Pest Management (IPM) involves utilizing all possible techniques to keep pest populations below a level causing economic injury (Dent, 1995). While the herbal extracts examined here did not show 100% success as prophylactic or curative treatments, the observed efficacy of these extracts lends support for their use in controlling the impact of monogenean infections on cultured fish. Overall, the results of these trials suggest that dietary supplementation of rosemary and garlic should be considered as options in the IPM of monogeneans in yellowtail kingfish production. While individually the rosemary and garlic compounds were effective at reducing the abundance of *Z. seriolae* infections, the potential therapeutic treatment of monogenean infections may prove to be more effective when both are used in tandem and in addition to other treatments. Furthermore, a practical application of incorporating these treatments in fish diet for commercial on-farm supplementation may be to coat the various herbal products externally onto feed pellets using 15 mL/kg of fish oil, as opposed to the lengthy procedure of grinding and reconstructing pellets with the various herbal supplements. Longer-term studies in the future may prove beneficial not only to better determine the effects these herbs have on growth for trial periods longer than 30 days, but also to identify the best IPM strategy to combat monogeneans in yellowtail kingfish aquaculture.

Acknowledgements

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Appendix 8 – Joan Gao salinity report

Physiological impacts of low-salinity bathing on yellowtail kingfish (*Seriola lalandi*) as a potential treatment for monogenean parasites

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Introduction

Parasite infections and associated diseases are major production-limiting factors impacting fish aquaculture worldwide. Parasite infections have the ability to cause significant mortality when left untreated and lead to susceptibility of fish to secondary infections (Leef and Lee, 2009). Parasite treatment and prevention in aquaculture is labour intensive and costly, and reinfection often occurs soon after treatment, particularly with parasites such as monogeneans which have direct life-cycles (Bravo et al., 2010). Thus, detailed understanding of parasite and host ecology and physiological limitations is integral to successful parasite management on commercial aquaculture farms (Brazenor and Hutson, 2015).

Monogenean ectoparasites are generally controlled through direct chemical bath treatments such as hydrogen peroxide or praziquantel or using freshwater bathing for marine fish (Adams et al., 2012; Hirazawa et al., 2016; Sharp et al., 2004). Indirect controls often employed to control monogenean infestations involves breaking transmission by cleaning nets and desiccation to kill eggs (Ernst et al., 2005). However, it is largely unknown how these treatments impact on the physiology on fish, as most research focuses only on the efficacy of the treatment against the parasite. Whilst some research has been published on the effects of chemical bathing on marine fish physiology, the effects freshwater bathing on marine fish are less studied. Whilst data exist on the effects of freshwater bathing on Atlantic salmon farms for treatment against amoebic gill disease or sea lice (Arriagada et al., 2016; Marcos-Lopez et al., 2017), Atlantic salmon are anadromous and have the physiology that allows them to rapidly adapt to both fresh and seawater.

Several species of the Seriola genus are farmed throughout the world and in all farming locations are susceptible to monogenean parasites including *Zeuxapta seriolae*, *Benedenia*

seriolae and *Neobenedenia girellae*. Whilst it is known that these parasites are susceptible to freshwater bathing, there is no data to show how well their host tolerates such low salinity. The aim of these trials was therefore to understand the physiological impacts of low-salinity bathing for periods of time relevant for controlling monogeneans infections on yellowtail kingfish, *Seriola lalandi*. Freshwater treatment is commonly used in marine fish and Atlantic salmon aquaculture against ectoparasites such as monogeneans, amoebic gill disease and sea lice (Adams et al., 2012; Fajer-Ávila et al., 2008; Harris et al., 2005; Ogawa, 2015; Powell et al., 2015).

Materials and Methods

Animal Ethics

All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes. All animal husbandry and experimental methods were approved by the Curtin University Animal Ethics Committee under permit ARE2017-14. Yellowtail kingfish were sourced from the Australian Centre for Applied Aquaculture Research (ACAAR) in Fremantle, Western Australia.

Salinity Trial 1

The first salinity trial tested the effect of four different salinity levels (0, 2, 5 and 35ppt) for three bathing durations (10, 30 and 60 mins) in large (2977 ± 71 grams) fish. The trial was run over 2 days, testing two salinities per day. Six, 1000L tanks were used. Sodium thiosulphate and aeration was added to dechlorinate the freshwater used to dilute seawater to the experimental salinity and held overnight prior to the introduction of fish. Each tank was stocked with 9 microchipped fish (without salinity acclimation). After 10, 30 and 60 minutes 3 pseudo-replicate fish were removed from each tank. Following the blood sampling described below fish were then transferred back to seawater (without acclimation) and monitored for 48 hours.

Salinity Trial 2

The second salinity trial tested the same salinities and bathing durations as Trial 1 but used smaller fish (362 ± 12 grams). Six x 350L tanks were used for this trial and filled to 100L to maintain a similar stocking density as the previous trial.

Blood collection and osmolality analysis

After the allocated bathing intervals in each of the above salinity trials, fish were individually anaesthetised (20 mg/L AQUI-S at the same salinity as the experimental treatment) and a blood sample taken from the caudal peduncle with a lithium-heparinised needle and syringe The blood was then transferred to a lithium-heparinised blood tube and stored in an ice slurry. Blood samples were then centrifuged at 10,000 rpm for 4 mins at 4ºC and the plasma transferred into tubes and frozen at -20°C until analysis. Plasma osmolality was analysed with a cryopscopic osmoteter (Osmomat 0.30, Gonotec). The remaining plasma was analysed for sodium, chloride and protein by VetPath. Plasma samples from 0 ppt and 35 ppt treatments after the 60 minutes bathe duration were also analysed for cortisol.

Statistical analyses

All data analysis undertaken by two-way ANOVA, with salinity and time set as factors. Size was not considered as a third factor, as the large and small fish were from two different cohorts. Post-hoc analysis was undertaken to find significance within each treatment factor and for interaction effect, $P < 0.05$ was accepted as significantly different.

Results

Salinity Trial Mortalities

In the large fish trial, one fish in the 0 ppt, 60-minute treatment died within 1 hour of the trial ending and another the next day, yielding 67% mortality in this treatment. No fish in any other treatment in either trial died.

Osmolality

In large fish, there was a significant effect of salinity ($P<0.0001$), bathe time ($P = 0.04$) and the interaction of these terms ($P = 0.04$) on osmolality. Those fish bathed at 0 ppt had significantly lower osmolality (LSM = 380 mOsm/kg) than all other treatments, which did not differ from each other (range 420 to 448 mOsm/kg) (**Error! Reference source not found.**). Osmolality of

plasma in these fish held at 0 ppt salinity was not significantly different to the control after 10 minutes, but dropped thereafter, suggesting that this short duration was well tolerated. Osmolality in the plasma of fish exposed to 0 ppt for 60 minutes was 343 ± 15 mOsm/kg and two of these three fish subsequently died, demonstrating that this level of osmolality is indicative of fatal disruption to osmoregulatory capacity.

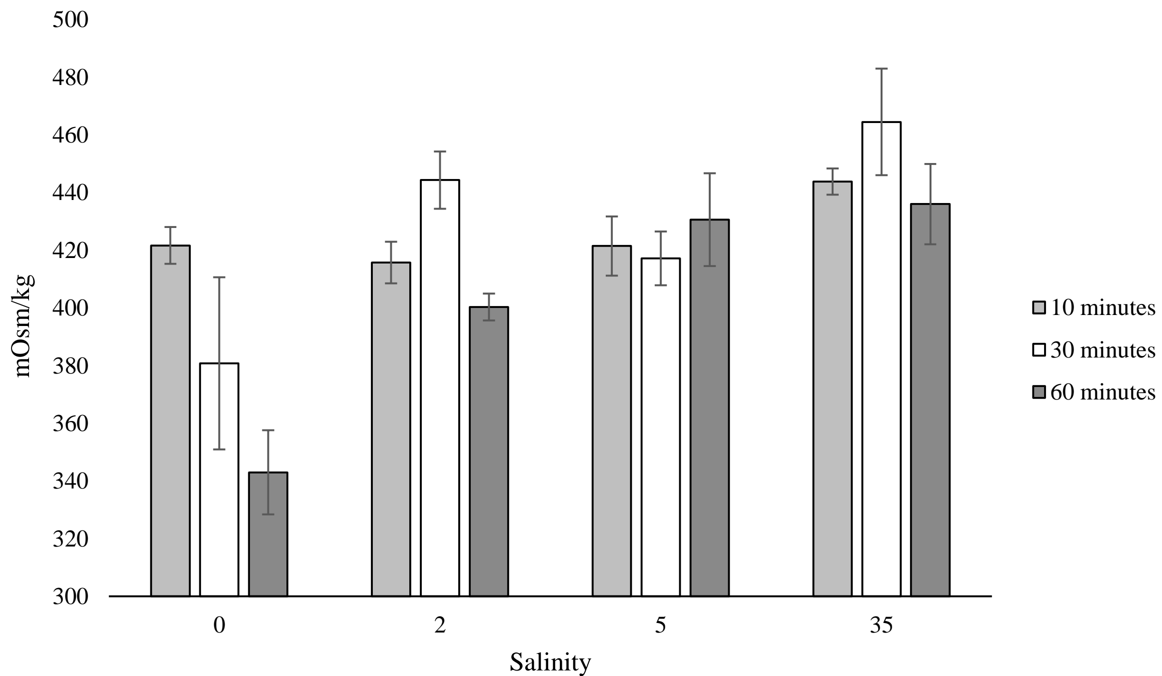

Figure 1: Effect of salinity and duration of exposure to these salinities on the osmolality of plasma in large YTK.

Small fish showed a similar response to salinity and bathe time, with osmolality dropping to <400 mM after 30 and 60 minutes in freshwater, however two-way ANOVA showed there to be no effect of bathe time of salinity on osmolality (Figure 2). One-way ANOVA on the 0 ppt data alone showed a significant effect of bathe time on osmolality ($P = 0.01$), with those fish bathed for 30 and 60 minutes have significantly lower osmolality than those bathed for 10 minutes. That osmolality did not drop as low in small fish as in large fish in the 0 ppt salinity suggests that smaller fish may be more tolerant to low salinity than large fish.

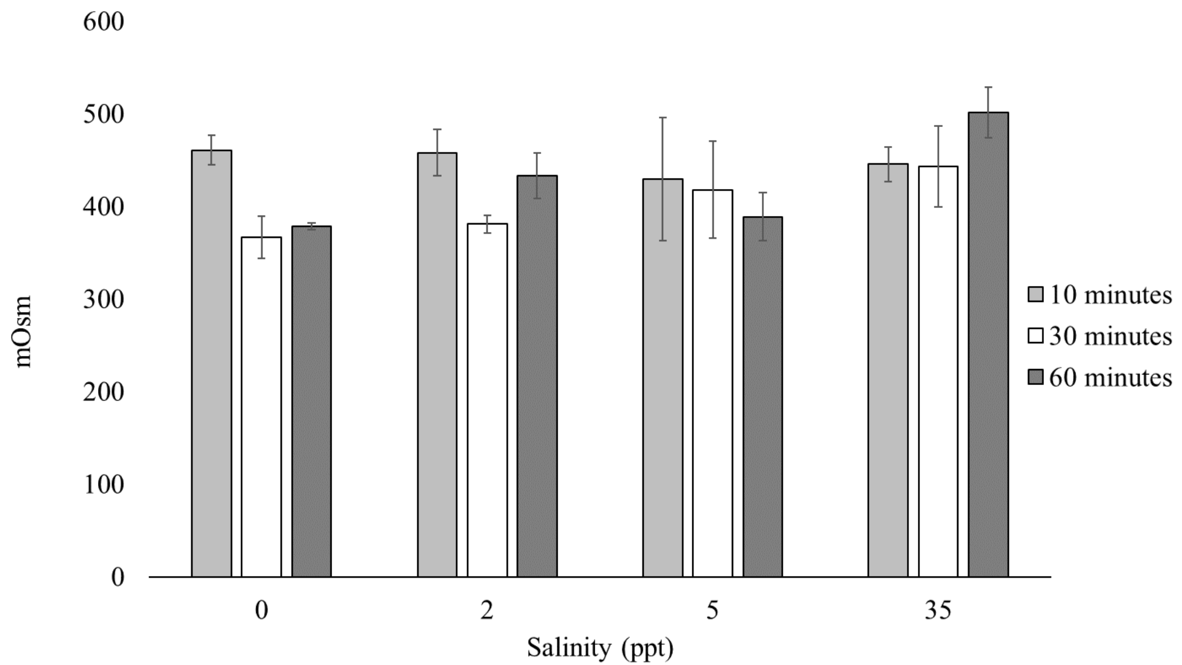

Figure 2: Effect of salinity and duration of exposure to these salinities on the osmolality of plasma in small YTK.

Plasma Sodium and Chloride Concentrations

As expected, the osmotic disruption noted in the osmolality data was also reflected in similar significant reductions in both sodium and chloride (the major electrolytes contributing to osmolality).

Large fish in freshwater (0 ppt) had significantly lower plasma sodium levels than those held at low salinities (2 and 5 ppt) and in the control (35 ppt) (p = 0.02, 0.003 and <0.001, respectively) (Figure 3).

Salinity also affected small yellowtail kingfish plasma sodium levels (P < 0.0001), where the control salinity (35 ppt) was significantly different from low salinity treatments (p < 0.001 for 0, 2 and 5 ppt). Sodium was significantly different between treatment times for large and small yellowtail kingfish (P =0.01 and <0.001, respectively), with a bathe time of 60 mins resulting in significantly lower sodium levels than 10 and 30 mins.

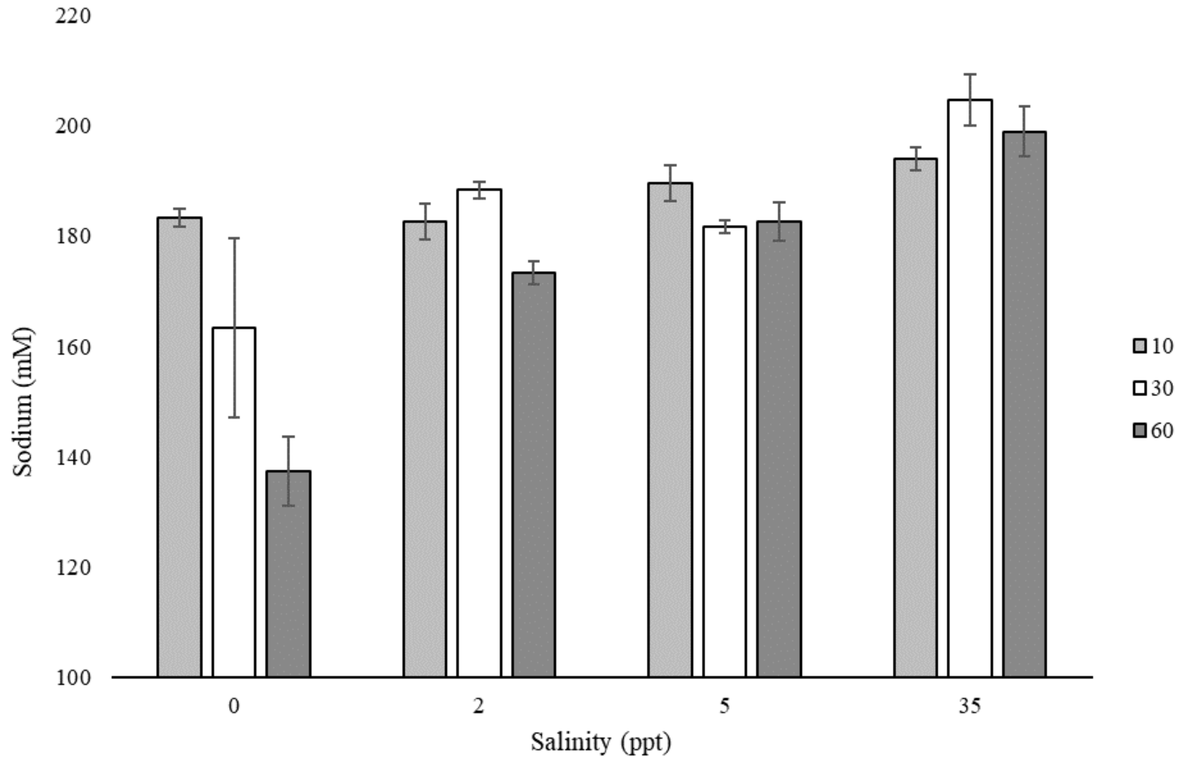

Figure 3: Effect of salinity and duration of exposure to these salinities on the concentration of sodium in the plasma of large YTK.

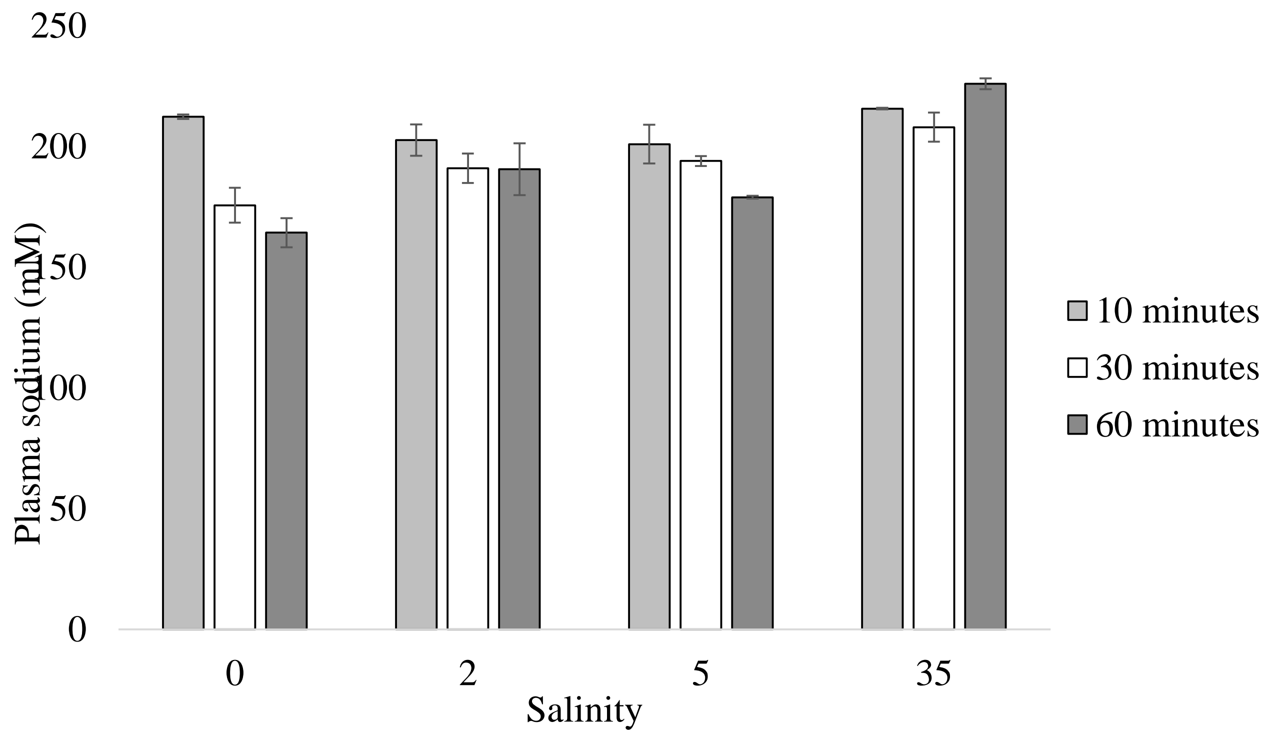

Figure 4: Effect of salinity and duration of exposure to these salinities on the concentration of sodium in the plasma of small YTK.

Similar to plasma sodium, longer bathe times (30 and 60 mins) were found to have a greater effect on plasma chloride than the shorter bathe time of 10 mins, for both sized fish (Figures 5 and 6).

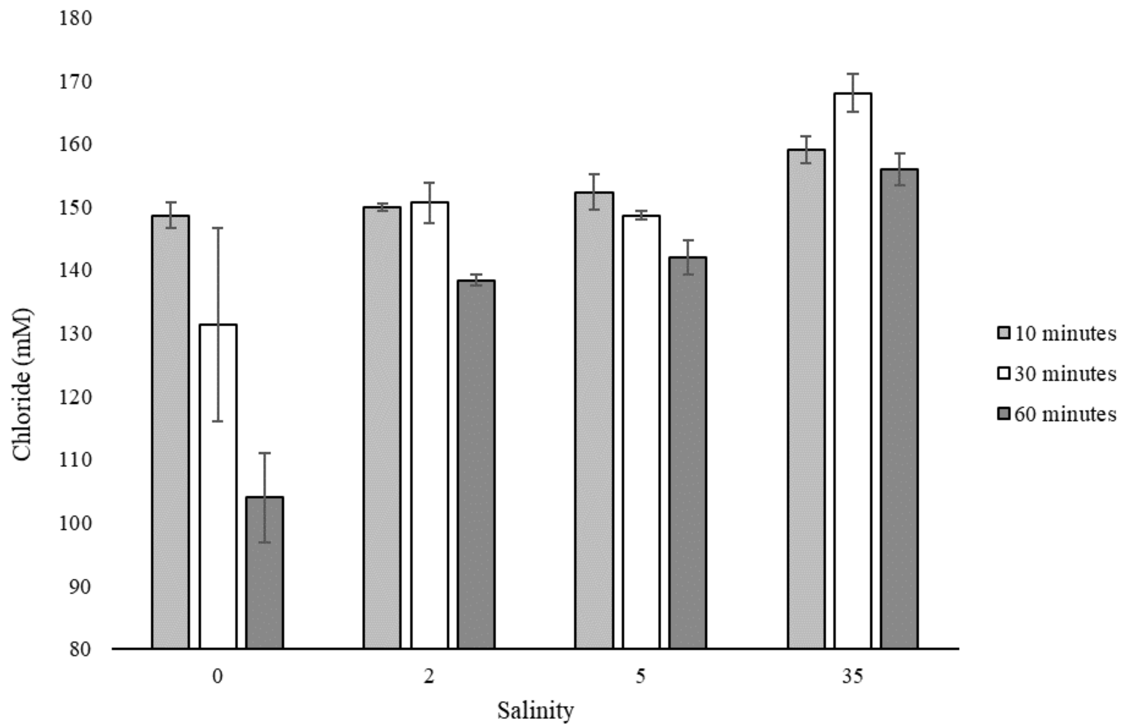

Figure 5: Effect of salinity and duration of exposure to these salinities on the concentration of chloride in the plasma of large YTK.

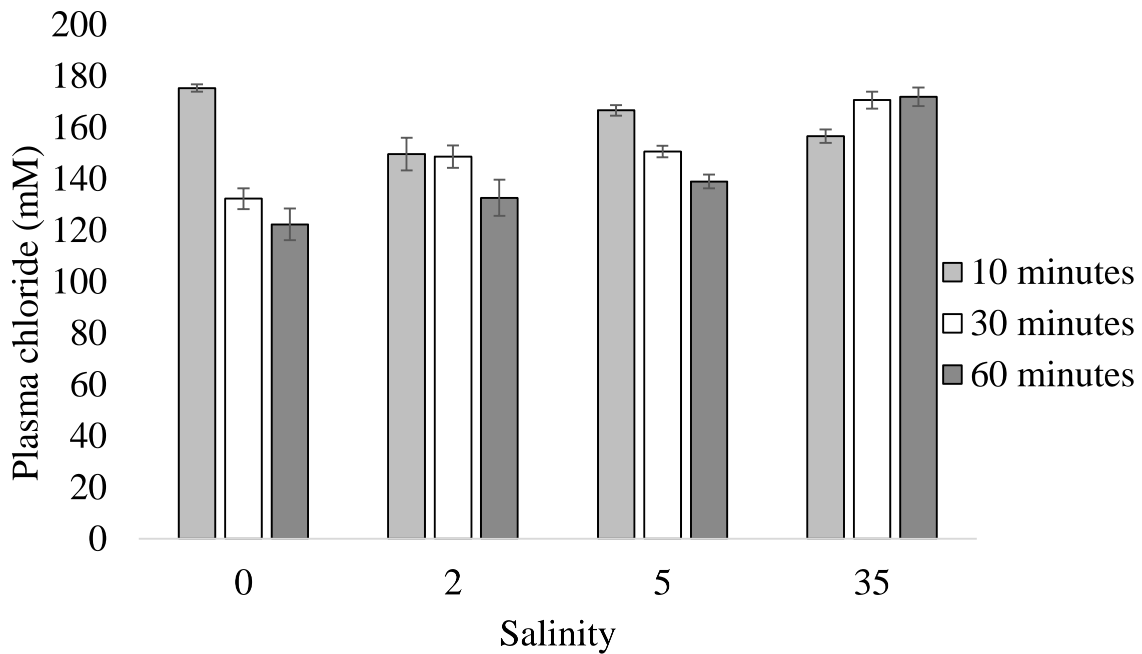

Figure 6: Effect of salinity and duration of exposure to these salinities on the concentration of chloride in the plasma of large YTK.

Plasma protein

The response to salinity and bathe time was similar between large (Figure 7) and small (Figure 8) fish and was consistent with the findings outlined above for osmoregulatory function. After 10 minutes of bathing in freshwater there was no difference in plasma protein from the control treatment, supporting the findings above that this short period of exposure to freshwater is well tolerated. However, after 30 and 60 minutes, plasma protein in fish bathed in freshwater was significantly higher ($P < 0.01$) than those in the 35 ppt control treatment from the same bathing duration. There were no significant differences in plasma protein between fish bathed at 2, 5 and 35 ppt at any time point in large and small fish, again demonstrating that the low salinities are well tolerated for up to 60 minutes.

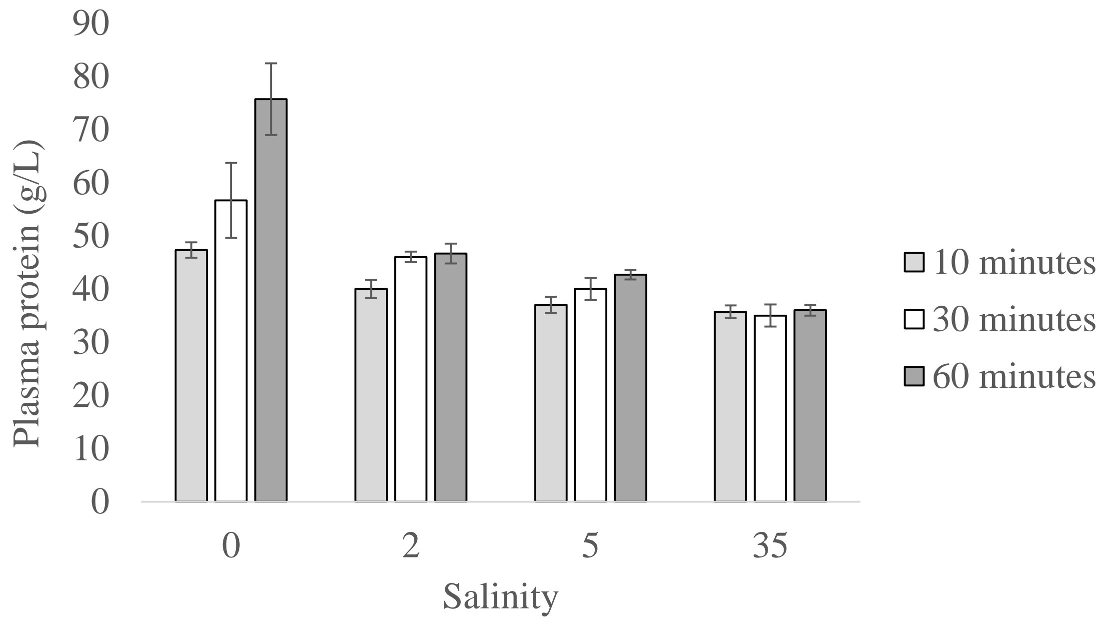

Figure 7: Effect of salinity and duration of exposure to these salinities on the plasma protein content of plasma in large YTK.

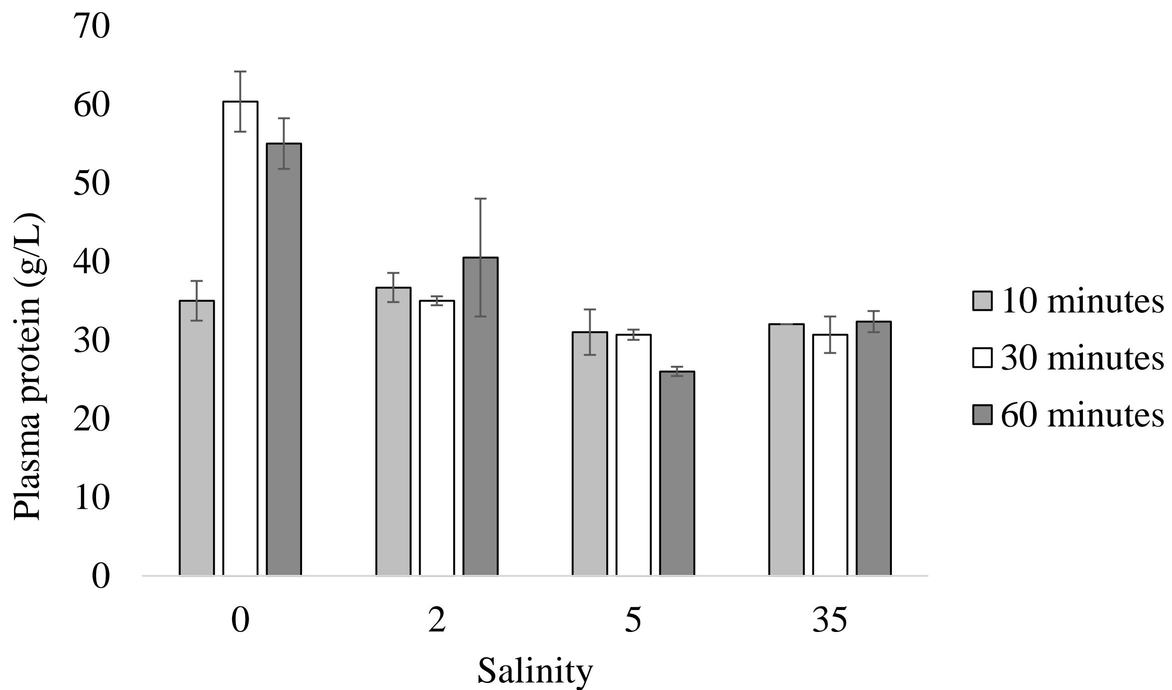

Figure 8: Effect of salinity and duration of exposure to these salinities on the plasma protein content of plasma in small YTK.

Cortisol levels

Cortisol levels doubled in large fish held in freshwater (814 ± 149 nmol/L) for 60 minutes compared with those held at 35 ppt at 0 ppt (402 ± 85 nmol/L), however this increase was not significant (P = 0.07) (Figure 9). Small fish also showed an increase in cortisol levels at 0 ppt (529 ± 19 nmol/L) compared to 35 ppt (461 ± 18 nmol/L); a difference that also wasn't significant (P = 0.09)(Figure 10).

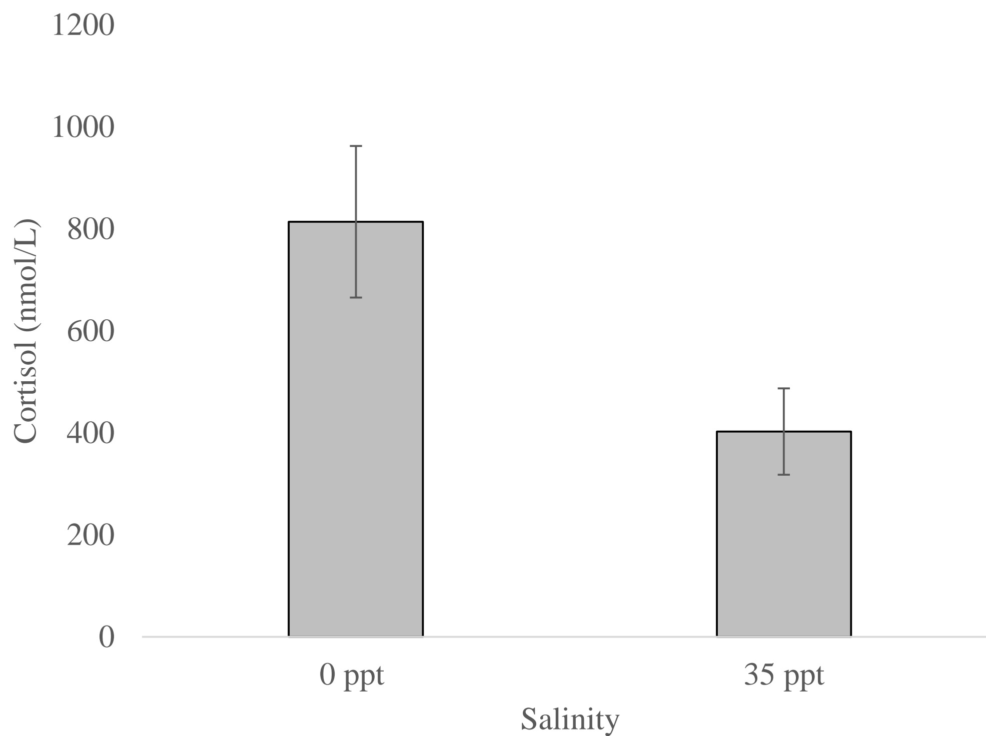

Figure 9: Effect of exposure to two salinities for 60 minutes on the plasma cortisol content of plasma in large YTK.

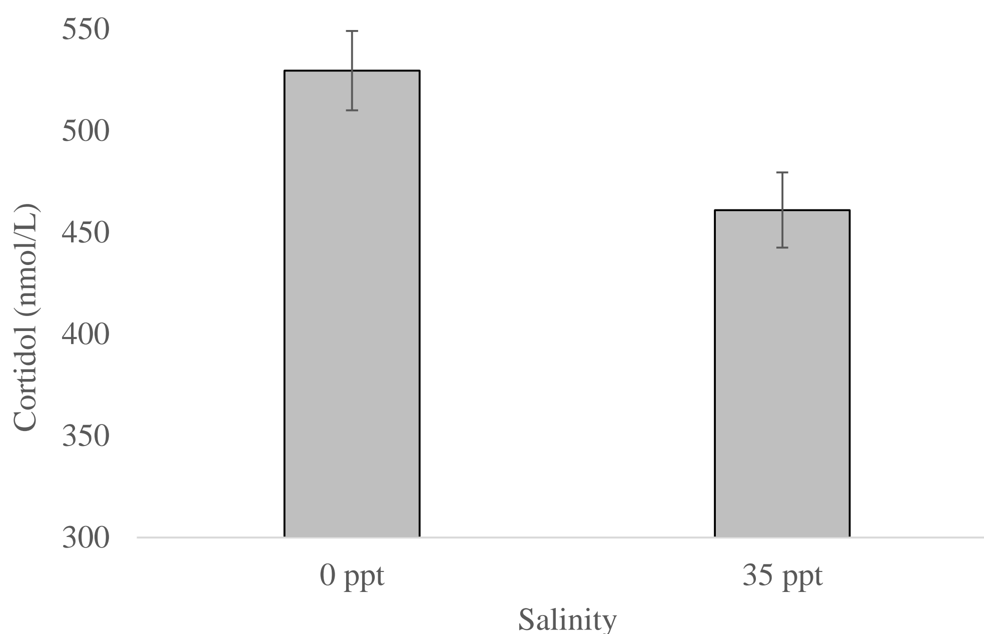

Figure 10: Effect of exposure to two salinities for 60 minutes on the plasma cortisol content of plasma in small YTK.

Discussion

Yellowtail kingfish are pelagic marine fish thus it was expected low salinity and freshwater would have a physiological effect, but how much of an affect was unknown and this study was conducted to understand these effects. Fish bathed in freshwater (0 ppt) presented with higher cortisol levels compared to the control treatment (35 ppt) at 60 mins. Interestingly, freshwater appeared to have a greater effect on large yellowtail kingfish in terms of cortisol levels compared to small yellowtail kingfish. The difference in effects could be due to the developmental process, as juvenile yellowtail kingfish tend to congregate in the shallow coastal waters where temperature and salinity are subject to greater change (Sampaio and Bianchini, 2002), through water runoff from land and flooding (Magill and Sayer, 2004). This could explain the small yellowtail kingfish tolerance to freshwater compared to the large yellowtail kingfish.

Yellowtail kingfish are osmoregulators, that means they actively regulate ions through their gills, stomach and kidneys (McCormick, 2001) and at full strength seawater (30 – 35 ppt) a lot of energy is required to maintain osmotic balance (Blanco Garcia et al., 2015). The general osmolality trend in this current study is as salinity decreases and time increases, plasma

osmolality decreases. This osmotic imbalance can be explained by non-adaptation of low salinities to yellowtail kingfish or other marine fish physiology (Sampaio and Bianchini, 2002).

Cortisol is one of the hormones that regulate ions, such as sodium, potassium and chloride for marine fish. Sodium is one element that marine teleosts must actively remove from their body to maintain plasma osmolality and for most fishes that means the decrease in salinity also means the decrease in sodium levels due to their natural physiology (Imsland et al., 2008). As it was the case in this study, where sodium concentration in plasma was significantly lower in the freshwater at 60 mins treatment. Similar results were found in Imsland et al. (2008) study on juvenile Atlantic halibut, where fish reared in 15 and 20 ppt had lower sodium concentration compared to fish reared in 32 ppt. Low salinity (2 and 5 ppt) decreased sodium concentrations when compared to the control group (35 ppt), but the difference was greater between freshwater and the control group. This suggests that 2 and 5 ppt has less of an effect on sodium concentrations and can potentially be used commercially as a treatment against monogeneans and other ecto-parasites.

Chloride concentrations display similar patterns compared to sodium, as they are found in similar levels within the marine environment. Chloride concentration between the control group and low salinities across all times were found to be significantly different, however the difference in concentrations was minimal.

Knowing low salinities (2 and 5 ppt) at 30 and 60 mins has less of a pathophysiological impact on yellowtail kingfish, when compared to freshwater at 30 and 60 mins. It is currently unclear as to how low salinity would affect the time at which death will occur for species of monogeneans infecting *Seriola lalandi*. Freshwater bathing is used as a treatment against sea lice, amoebic gill disease and monogeneans (Ernst et al., 2005; Fajer-Ávila et al., 2008; Powell et al., 2015; Taylor et al., 2009). Skin flukes generally die and detach within minutes after freshwater treatment (Brazenor and Hutson, 2015; Ernst et al., 2005). However, freshwater bathing may be less effective at killing some species of gill flukes due to their ability to shield themselves from direct exposure by embedding deeply in gill lamellae and mucous. Further investigation on monogenean survival rates at 2 and 5 ppt can be used to assess treatment options for commercial aquaculture farms.

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